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ANTI-GLYPICAN 3 ANTIBODY

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to an anti-glypican 3 antibody, a cell growth inhibitor and an anticancer agent containing the antibody as an active ingredient.

Description of Related Art

Glypican 3 (GPC3) is one of the glypican family of heparan sulfate proteoglycans that are present on cell surfaces. It is suggested that GPC3 may be involved in cell division in development or cancer cell growth, however, its function has not been well elucidated yet.

It has been found that a certain type of antibody binding to GPC3 has a cell growth-inhibiting activity via an antibody-dependent cell-mediated cytotoxicity (ADCC) activity and a complement-dependent cytotoxicity (CDC) activity (International Patent Application WO 2003/000883). In addition, it has been suggested that GPC3 is cleaved in vivo and secreted into blood as a secreted form of GPC3, and the diagnosis of cancers may be possible by using an antibody capable of detecting the secreted form of GPC3 (International Patent Applications WO 2004/022739, WO 03/100429 and WO

2004/018667).

When developing an anticancer agent based on the cytotoxicity activity of an antibody, it is preferred that the antibody to be used has high ADCC activity or CDC activity. Accordingly, an anti-GPC3 antibody having a high cytotoxicity activity has been desired as an antibody recognizing GPC3.

An object of the present invention is to provide an anti-GPC3 antibody having a higher ADCC activity and CDC activity compared with those of a conventional antibody.

SUMMARY OF THE INVENTION

The present inventors have succeeded in obtaining an antibody having a higher cytotoxicity activity compared with that of a conventional anti-glypican 3 antibody. Furthermore, they analyzed epitopes for such an antibody and succeeded in determining the regions on GPC 3 recognized by the antibody with a high cytotoxicity activity.

In one aspect, the present invention provides an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 of any one of (1) - (12):

(1) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 123, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 124, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 125;

(2) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 109, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 110, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 111;

(3) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 106, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 107, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 108;

(4) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 132, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 133, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 134;

(5) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 106, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 135, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 136;

(6) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 126, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 127, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 128;

(7) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 129, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 130, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 131;

(8) CDR1 comprising the amino acid sequence set forth in SEQ

ID NO: 103, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 104, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 105;

(9) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 118, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 121, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 122;

(10) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 115, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 116, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 117;

(11) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 112, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 113, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 114; or

(12) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 118, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 119, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 120.

In another aspect, the invention provides an antibody comprising a light chain variable region having CDRs 1, 2 and 3 of any one of (1) - (13):

(1) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 143, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 144, and CDR3 comprising the amino acid sequence

set forth in SEQ ID NO: 158;

(2) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 143, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 144, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 145;

(3) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 140, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 141, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 142;

(4) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 167, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 168, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 169;

(5) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 170, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 144, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 171;

(6) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 159, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 160, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 161;

(7) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 162, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 147, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 163;

(8) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 164, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 165, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 166;

(9) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 137, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 138, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 139;

(10) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 155, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 156, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 157;

(11) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 149, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 150, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 151;

(12) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 146, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 147, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 148; or

(13) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 152, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 153, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 154.

Preferably, the antibody of the invention is selected

from the group consisting of any one of (1) - (13):

(1) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 123, 124 and 125, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 143, 144 and 158, respectively;

(2) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 109, 110 and 111, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 143, 144 and 145, respectively;

(3) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 106, 107 and 108, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 140, 141 and 142, respectively;

(4) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 132, 133 and 134, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 167, 168 and 169, respectively;

(5) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 106, 135 and 136, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 170, 144 and 171, respectively;

(6) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 126, 127 and 128, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 159, 160 and 161, respectively;

(7) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 129, 130 and 131, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 162, 147 and 163, respectively;

(8) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 129, 130 and 131, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 164, 165 and 166, respectively;

(9) an antibody comprising a heavy chain variable region

having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 103, 104 and 105, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 137, 138 and 139, respectively;

(10) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 118, 121 and 122, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 155, 156 and 157, respectively;

(11) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 115, 116 and 117, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 149, 150 and 151, respectively;

(12) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 112, 113 and 114, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 146, 147 and 148, respectively; and

(13) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set

forth in SEQ ID NO: 118, 119 and 120, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 152, 153 and 154, respectively.

In another aspect, the invention provides an antibody having a heavy chain variable region of any one of (1) - (7):

(1) a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 84;

(2) a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 85;

(3) a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 86;

(4) a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 87;

(5) a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 88;

(6) a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 89; or

(7) a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 90.

In another aspect, the invention provides an antibody having a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 92.

Preferably, the antibody of the invention is selected from the group consisting of the antibody of any one of (1)

- (7):

(1) an antibody comprising a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 84 and a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 92;

(2) an antibody comprising a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 85 and a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 92;

(3) an antibody comprising a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 86 and a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 92;

(4) an antibody comprising a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 87 and a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 92;

(5) an antibody comprising a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 88 and a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 92;

(6) an antibody comprising a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 89 and a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 92; and

(7) an antibody comprising a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 90 and a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 92.

In another aspect, the invention provides an antibody comprising a light chain variable region having CDRs 1, 2 and 3 of any one of (1) - (15):

(1) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 174, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 144, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 158;

(2) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 175, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 144, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 158;

(3) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 176, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 144, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 158;

(4) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 177, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 144, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 158;

(5) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 178, CDR2 comprising the amino acid sequence set forth

in SEQ ID NO: 144, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 158;

(6) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 179, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 144, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 158;

(7) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 180, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 144, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 158;

(8) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 181, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 144, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 158;

(9) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 182, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 144, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 158;

(10) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 183, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 144, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 158;

(11) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 184, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 144, and CDR3 comprising the amino acid sequence

set forth in SEQ ID NO: 158;

(12) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 185, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 144, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 158;

(13) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 186, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 144, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 158;

(14) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 187, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 144, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 158; or

(15) CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 188, CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 144, and CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 158.

In another aspect, the invention provides an antibody selected from the group consisting of the antibody of (1) - (15):

(1) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 123, 124 and 125, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 174, 144 and 158,

respectively;

(2) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 123, 124 and 125, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 175, 144 and 158, respectively;

(3) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 123, 124 and 125, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 176, 144 and 158, respectively;

(4) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 123, 124 and 125, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 177, 144 and 158, respectively;

(5) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 123, 124 and 125, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 178, 144 and 158, respectively;

(6) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 123, 124 and 125, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 179, 144 and 158, respectively;

(7) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 123, 124 and 125, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 180, 144 and 158, respectively;

(8) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 123, 124 and 125, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 181, 144 and 158, respectively;

(9) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 123, 124 and 125, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 182, 144 and 158, respectively;

(10) an antibody comprising a heavy chain variable region

having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 123, 124 and 125, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 183, 144 and 158, respectively;

(11) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 123, 124 and 125, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 184, 144 and 158, respectively;

(12) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 123, 124, and 125, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 185, 144, and 158, respectively;

(13) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 123, 124, and 125, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 186, 144 and 158, respectively;

(14) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set

forth in SEQ ID NO: 123, 124 and 125, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 187, 144 and 158, respectively; and

(15) an antibody comprising a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 123, 124 and 125, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 188, 144 and 158, respectively.

In further aspect, the invention provides an antibody having a light chain variable region selected from (1) - (15):

(1) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 191;

(2) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 192;

(3) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 193;

(4) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 194;

(5) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 195;

(6) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 196;

(7) a light chain variable region comprising the amino acid

sequence set forth in SEQ ID NO: 197;

(8) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 198;

(9) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 199;

(10) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 200;

(11) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 201;

(12) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 202;

(13) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 203;

(14) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 204; and

(15) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 205.

In another aspect, the invention provides an antibody having a light chain variable region selected from the group consisting of (1) - (15):

(1) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 191;

(2) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 192;

(3) a light chain variable region comprising the amino acid

sequence set forth in SEQ ID NO: 193;

(4) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 194;

(5) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 195;

(6) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 196;

(7) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 197;

(8) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 198;

(9) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 199;

(10) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 200;

(11) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 201;

(12) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 202;

(13) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 203;

(14) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 204; and

(15) a light chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 205;

and a heavy chain variable region selected from the group consisting of (1) - (7):

(1) a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 84;

(2) a heavy chain variable region comprising; the amino acid sequence set forth in SEQ ID NO: 85;

(3) a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 86;

(4) a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 87;

(5) a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 88;

(6) a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 89; and

(7) a heavy chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 90.

The heavy chain variable region, a light chain variable region, and the amino acid sequence of the CDRs 1, 2 and 3, as well as the SEQ ID NOs are summarized in the table below.

Antibody and variable regions		SEQ ID NO
M3C11	H	22
M13B3	H	23
M1E7	H	24
M3B8	H	25
M11F1	H	26
M19B11	H	27
M6B1	H	28
M18D4	H	29

M5B9	H	30
M10D2	H	31
L9G11	H	32
M3C11	L	44
M13B3	L	45
M1E7	L	46
M3B8	L	47
M11F1	L	48
M19B11	L	49
M6B1	L	50
M18D4	L	51
M5B9	L	52
M10D2	L	53
L9G11	L	54
GC199	H	60
GC202	H	61
GC33	H	62
GC179	H	63
GC194	H	64
GC199	L	71
GC202	L	72
GC33	L	73
GC179	L	74
GC194(1)	L	75
GC194(2)	L	76
GC33.ver.a	H	84
GC33.ver.c	H	85
GC33.ver.f	H	86
GC33.ver.h	H	87
GC33.ver.i	H	88
GC33.ver.j	H	89
GC33.ver.k	H	90
GC33.ver.a	L	92
M13B3(H)	CDR1	103
	CDR2	104
	CDR3	105
M3B8(H)	CDR1	106
	CDR2	107
	CDR3	108
M11F1(H)	CDR1	109
	CDR2	110
	CDR3	111
M5B9(H)	CDR1	112
	CDR2	113
	CDR3	114

M6B1(H)	CDR1	115
	CDR2	116
	CDR3	117
M10D2(H)	CDR1	118
	CDR2	119
	CDR3	120
L9G11(H)	CDR1	118
	CDR2	121
	CDR3	122
GC33(H)	CDR1	123
	CDR2	124
	CDR3	125
GC179(H)	CDR1	126
	CDR2	127
	CDR3	128
GC194(H)	CDR1	129
	CDR2	130
	CDR3	131
GC199(H)	CDR1	132
	CDR2	133
	CDR3	134
GC202(H)	CDR1	106
	CDR2	135
	CDR3	136
M13B3(L)	CDR1	137
	CDR2	138
	CDR3	139
M3B8(L)	CDR1	140
	CDR2	141
	CDR3	142
M11F1(L)	CDR1	143
	CDR2	144
	CDR3	145
M5B9(L)	CDR1	146
	CDR2	147
	CDR3	148
M6B1(L)	CDR1	149
	CDR2	150
	CDR3	151
M10D2(L)	CDR1	152
	CDR2	153
	CDR3	154
L9G11(L)	CDR1	155
	CDR2	156
	CDR3	157

GC33(L)	CDR1	143
	CDR2	144
	CDR3	158
GC179(L)	CDR1	159
	CDR2	160
	CDR3	161
GC194(L)1	CDR1	162
	CDR2	147
	CDR3	163
GC194(L)2	CDR1	164
	CDR2	165
	CDR3	166
GC199(L)	CDR1	167
	CDR2	168
	CDR3	169
GC202(L)	CDR1	170
	CDR2	144
	CDR3	171
GC33(L)	G34A	174
GC33(L)	G34D	175
GC33(L)	G34E	176
GC33(L)	G34F	177
GC33(L)	G34H	178
GC33(L)	G34N	179
GC33(L)	G34P	180
GC33(L)	G34Q	181
GC33(L)	G34I	182
GC33(L)	G34K	183
GC33(L)	G34L	184
GC33(L)	G34V	185
GC33(L)	G34W	186
GC33(L)	G34Y	187
GC33(L)	G34R	188

Also the invention features an antibody having an activity equivalent to the activity of the antibody described above, wherein one or more amino acid residues are substituted, deleted or added and/or inserted from the amino acid sequences described above.

Preferably, the antibody of the invention is a humanized

antibody.

Thus, in another aspect, the invention provides a humanized antibody capable of binding to glypican 3.

In further aspect, the invention provides an antibody capable of binding to a peptide consisting of the sequence of the amino acid residues 524 - 563 of glypican 3.

Preferably, the antibody of the invention is capable of binding to a peptide consisting of the sequence of the amino acid residues 537 - 563 of glypican 3. More preferably, the antibody of the invention does not bind to a peptide consisting of the sequence of the amino acid residues 550 - 563 of glypican 3.

Preferably, the antibody is capable of binding to a peptide consisting of the sequence of the amino acid residues 544 - 553 of glypican 3 or a peptide consisting of the sequence of the amino acid residues 546 - 551 of glypican 3.

In still another aspect, the invention provides an antibody capable of binding to an epitope to which a second antibody is capable of binding, wherein said second antibody comprises a heavy chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 123, 124 and 125, respectively, and a light chain variable region having CDRs 1, 2 and 3 comprising the amino acid sequence set forth in SEQ ID NO: 143, 144 and 158, respectively. Namely, the antibody of the invention is capable of competing in binding

to GPC3 with the second antibody.

In a preferred embodiment, the antibody of the invention is capable of binding to glypican 3 and has a high CDC activity against a cell expressing glypican 3 and/or has a high ADCC activity against a cell expressing glypican 3.

In another aspect, the invention provides a polynucleotide coding for a heavy chain variable region or a light chain variable region of the antibody of the invention.

Preferably, the polynucleotide of the invention has the sequence set forth in SEQ ID NOs: 11-21, 33-43, 55-59, 65-70 and 77-83.

In still another aspect, the invention provides a cell-growth inhibitor and an anticancer agent comprising as an active ingredient the antibody of the invention. Preferably, the anticancer agent of the invention is used for treatment of hepatoma.

In further aspect, the invention provides a peptide comprising the sequence of the amino acid residues 524 - 563 of glypican 3, the sequence of the amino acid residues 537 - 563 of glypican 3, the sequence of the amino acid residues 544 - 553 of glypican 3 or the amino acid sequence of the amino acid residues 546 - 551 of glypican 3.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the binding activity of the anti-GPC3

antibody to a CHO cell, a CHO cell expressing full-length GPC3, HepG2 and HuH-7, which was evaluated by flow cytometry. M1E7 (solid line) and M11F1 (dashed line) were used at a concentration of 5 μg/mL, respectively.

Fig. 2 is a table showing the results of epitope classification by a competitive ELISA. The degrees of competitive inhibition against the binding of the biotinylated anti-GPC3 antibody are indicated by percentage. The epitopes were classified into 5 groups, a to e, according to the competitive inhibition pattern.

Fig. 3 shows the results of evaluating by Western blotting whether an anti-GPC3 antibody binds to the N-terminal fragment of 40 kDa of the soluble form of GPC3 core protein or to the C-terminal fragment of 30 kDa thereof. It was found that L9G11 binds to the N-terminal fragment and M3C11 binds to the C-terminal fragment.

Fig. 4 shows the results of detecting a secreted form of GPC3 is present in the culture supernatant of HepG2 by a sandwich ELISA. It was strongly detected with the combination of antibodies that bind to the N-terminal fragment such as M6B1, M18D4 or M19B11, and it was not strongly detected with an antibody that binds to the C-terminal fragment such as M3C11, M13B3 or M3B8.

Fig. 5 shows the results of immunoprecipitation of the culture supernatant of HepG2 with the use of an anti-GPC3

antibody and detecton of a secreted form of GPC3. The medium as a control (lanes 1 and 3) and the culture supernatant of HepG2 (lanes 2 and 4) were immunoprecipitated using M1E7 (lanes 1 and 2) and M10D2 (lanes 3 and 4). Secretory GPC3 was detected by M10D2 that binds to the N-terminal fragment.

Fig. 6 shows the results of analyzing the epitope of the antibodies that bind to the C-terminal fragment of GPC3 by Western blotting with the use of a fusion protein of the C-terminal peptide of GPC3 and GST. The soluble form of GPC3 core protein (lane 1), GST (lane 2), GC-1 (lane 3), GC-2 (lane 4), GC-3 (lane 5), GC-4 (lane 6) and GC-5 (lane 7) were subjected to SDS electrophoresis under reducing conditions, and detected by Western blotting using M3C11 and M11F1.

Fig. 7 shows the results of evaluating the CDC activity of the anti-GPC3 mouse-human chimeric antibody to a CHO cell that expresses GPC3.

Fig. 8 shows the results of evaluating the ADCC activity of the anti-GPC3 mouse-human chimeric antibody to a CHO cell that expresses GPC3 and HepG2.

Fig. 9 shows the results of evaluating the ADCC activity of GC33 to a human hepatoma cell line, HuH-7, using a mouse bone marrow-derived effector cell.

Fig. 10 shows the results of evaluating the antitumor activity of GC33 antibody to a mouse model transplanted with human hepatoma.

Fig. 11 shows the results of evaluating the CDC activity of the mouse-human chimeric antibody GC33 to a CHO cell that expresses GPC3.

Fig. 12 shows the results of evaluating the ADCC activity of the mouse-human chimeric antibody GC33 to HepG2.

Fig. 13 shows GPC3-derived sequences contained in GST-fusion proteins (GC-4, 5, 6, 7, 8, 9, 11, 12, 13 and 14) prepared for analyzing the epitope of GC33.

Fig. 14 shows the results of Western blotting with the use of GC33 after separating GST, GC-7, 8, 9, 11, 12, 13 and 14 by SDS-PAGE under reducing conditions.

Fig. 15 shows the results of evaluating the binding activity of humanized GC33 to GPC3 by an ELISA.

Fig. 16 shows an antibody panel which summarizes isotypes and the results of an ELISA, BIAcore, FACS, an epitope analysis and an immunoprecipitation test for clones derived from a mouse immunized with a soluble form of GPC3.

Fig. 17 shows an antibody panel in which isotypes and the results of an ELISA, FACS and an epitope analysis for clones derived from a mouse immunized with GC-3 are summarized.

Fig. 18 shows the results of evaluating the binding activity of the modified antibodies to the soluble form of GPC3 core protein by an ELISA. Gly34 located at CDR1 in a humanized GC33 L chain variable region was replaced with any of 17 amino acids other than Cys and Met.

Fig. 19 shows the results of evaluating the CDC activity of the mouse-human chimeric antibodies GC33, M3C11, and M1E7 to a CHO cell that expresses full-length GPC3.

Fig. 20 shows the results of evaluating the ADCC activity of the mouse-human chimeric antibodies GC33, M3C11, and M1E7 to a human hepatoma cell line SK-03 that expresses full-length GPC3.

DETAILED DESCRIPTION OF THE INVENTION

Antibody

The present invention provides antibodies described in the following (I) to (XI).

(I) An antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: in any of the following (1) to (12):

(1) SEQ ID NOs: 123, 124 and 125 (GC33),

(2) SEQ ID NOs: 109, 110 and 111 (M11F1),

(3) SEQ ID NOs: 106, 107 and 108 (M3B8),

(4) SEQ ID NOs: 132, 133 and 134 (GC199),

(5) SEQ ID NOs: 106, 135 and 136 (GC202),

(6) SEQ ID NOs: 126, 127 and 128 (GC179),

(7) SEQ ID NOs: 129, 130 and 131 (GC194),

(8) SEQ ID NOs: 103, 104 and 105 (M13B3),

(9) SEQ ID NOs: 118, 121 and 122 (L9G11),

(10) SEQ ID NOs: 115, 116 and 117 (M6B1),

(11) SEQ ID NOs: 112, 113 and 114 (M5B9), and

(12) SEQ ID NOs: 118, 119 and 120 (M10D2).

Among the antibodies described in (1) to (12), preferred are the antibodies described in (1) to (8), more preferred are the antibodies described in (1) to (5), and particularly preferred is the antibody described in (1). The antibodies described in (1) to (8) recognize the C-terminal peptide of glypican 3 (a peptide comprising the 374th amino acid to the 580th amino acid of glypican 3); and are useful as a therapeutic antibody. In addition, the antibodies described in (9) to (12) recognize the N-terminal peptide of glypican 3 (a peptide comprising from the 1st amino acid to the 373rd amino acid of glypican 3); and are useful as a diagnostic antibody.

(II) An antibody containing light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: in any of the following (1) to (13):

(1) SEQ ID NOs: 143, 144 and 158 (GC33),

(2) SEQ ID NOs: 143, 144 and 145 (M11F1),

(3) SEQ ID NOs: 140, 141 and 142 (M3B8),

(4) SEQ ID NOs: 167, 168 and 169 (GC199),

(5) SEQ ID NOs: 170, 144 and 171 (GC202),

(6) SEQ ID NOs: 159, 160 and 161 (GC179),

(7) SEQ ID NOs: 162, 147 and 163 (GC194 (1)),

(8) SEQ ID NOs: 164, 165 and 166 (GC194 (2)),

(9) SEQ ID NOs: 137, 138 and 139 (M13B3),

(10) SEQ ID NOs: 155, 156 and 157 (L9G11),

(11) SEQ ID NOs: 149, 150 and 151 (M6B1),

(12) SEQ ID NOs: 146, 147 and 148 (M5B9), and

(13) SEQ ID NOs: 152, 153 and 154 (M10D2).

Among the antibodies described in (1) to (13), preferred are the antibodies described in (1) to (8), more preferred are the antibodies described in (1) to (5), and particularly preferred is the antibody described in (1). The antibodies described in (1) to (8) recognize the C-terminal peptide of glypican 3 (a peptide comprising from the 374th amino acid to the 580th amino acid of glypican 3); and are useful as a therapeutic antibody. In addition, the antibodies described in (9) to (13) recognize the N-terminal peptide of glypican 3 (a peptide comprising from the 1st amino acid to the 373rd amino acid of glypican 3); and are useful as a diagnostic antibody.

(III) An antibody selected from the group consisting of the antibodies described in the following (1) to (13):

(1) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 123, 124 and 125, and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 143, 144 and 158 (GC33),

(2) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth

in SEQ ID NOs: 109, 110 and 111, and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 143, 144 and 145 (M11F1),

(3) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 106, 107 and 108, and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 140, 141 and 142 (M3B8),

(4) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 132, 133 and 134, and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 167, 168 and 169 (GC199),

(5) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 106, 135 and 136, and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 170, 144 and 171 (GC202),

(6) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 126, 127 and 128, and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 159, 160 and 161 (GC179),

(7) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth

in SEQ ID NOs: 129, 130 and 131, and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 162, 147 and 163 (GC194 (1)),

(8) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 129, 130 and 131, and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 164, 165 and 166 (GC194 (2)),

(9) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 103, 104 and 105, and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 137, 138 and 139 (M13B3),

(10) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 118, 121 and 122, and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 155, 156 and 157 (L9G11),

(11) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 115, 116 and 117, and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 149, 150 and 151 (M6B1),

(12) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth

in SEQ ID NOs: 112, 113 and 114, and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 146, 147 and 148 (M5B9),

(13) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 118, 119 and 120, and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 152, 153 and 154 (M10D2).

Among the antibodies described in (1) to (13), preferred are the antibodies described in (1) to (8), more preferred are the antibodies described in (1) to (5), and particularly preferred is the antibody described in (1). The antibodies described in (1) to (8) recognize the C-terminal peptide of glypican 3 (a peptide comprising from the 374th amino acid to the 580th amino acid of glypican 3); and are useful as a therapeutic antibody. In addition, the antibodies described in (9) to (13) recognize the N-terminal peptide of glypican 3 (a peptide comprising from the 1st amino acid to the 373rd amino acid of glypican 3); and are useful as a diagnostic antibody.

(IV) An antibody having a heavy chain variable region described in any of the following (1) to (7):

(1) a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 84 (GC33 VH ver.a),

(2) a heavy chain variable region containing the amino acid

sequence set forth in SEQ ID NO: 85 (GC33 VH ver.c),
(3) a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 86 (GC33 VH ver.f),
(4) a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 87 (GC33 VH ver.h),
(5) a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 88 (GC33 VH ver.i),
(6) a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 89 (GC33 VH ver.j), and
(7) a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 90 (GC33 VH ver.k).

Among the antibodies described in (1) to (7), particularly preferred are the antibodies described in (2) to (7).

(V) An antibody having a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 92 (GC33 VL ver.a).

(VI) An antibody selected from the group consisting of the antibodies described in the following (1) to (7):
(1) an antibody having a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 84 (GC33 VH ver.a) and a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 92 (GC33 VL ver.a),
(2) an antibody having a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 85 (GC33 VH

ver.c) and a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 92 (GC33 VL ver.a), (3) an antibody having a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 86 (GC33 VH ver.f) and a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 92 (GC33 VL ver.a), (4) an antibody having a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 87 (GC33 VH ver.h) and a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 92 (GC33 VL ver.a), (5) an antibody having a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 88 (GC33 VH ver.i) and a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 92 (GC33 VL ver.a), (6) an antibody having a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 89 (GC33 VH ver.j) and a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 92 (GC33 VL ver.a), and (7) an antibody having a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 90 (GC33 VH ver.k) and a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 92 (GC33 VL ver.a).

Among the antibodies described in (1) to (7), particularly preferred are the antibodies described in (2) to (7).

(VII) An antibody described in any of the following (1) to (15):

(1) an antibody containing light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 174, 144 and 158,

(2) an antibody containing light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 175, 144 and 158,

(3) an antibody containing light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 176, 144 and 158,

(4) an antibody containing light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 177, 144 and 158,

(5) an antibody containing light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 178, 144 and 158,

(6) an antibody containing light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 179, 144 and 158,

(7) an antibody containing light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 180, 144 and 158,

(8) an antibody containing light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 181, 144 and 158,

(9) an antibody containing light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 182, 144 and 158,

(10) an antibody containing light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 183, 144 and 158,

(11) an antibody containing light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 184, 144 and 158,

(12) an antibody containing light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 185, 144 and 158,

(13) an antibody containing light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 186, 144 and 158,

(14) an antibody containing light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 187, 144 and 158, and

(15) an antibody containing light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 188, 144 and 158.

Among the antibodies described in (1) to (15), preferred is the antibody described in (15).

(VIII) An antibody described in any of the following (1) to (15):

(1) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 123, 124 and 125 and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 174, 144 and 158,

(2) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 123, 124 and 125 and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 175, 144 and 158,

(3) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 123, 124 and 125 and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 176, 144 and 158,

(4) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 123, 124 and 125 and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 177, 144 and 158,

(5) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 123, 124 and 125 and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 178, 144 and 158,

(6) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 123, 124 and 125 and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 179, 144 and 158,

(7) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 123, 124 and 125 and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 180, 144 and 158,

(8) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 123, 124 and 125 and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 181, 144 and 158,

(9) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 123, 124 and 125 and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 182, 144 and 158,

(10) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 123, 124 and 125 and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 183, 144 and 158,

(11) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 123, 124 and 125 and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 184, 144 and 158,

(12) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 123, 124 and 125 and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 185, 144 and 158,

(13) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 123, 124 and 125 and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 186, 144 and 158,

(14) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 123, 124 and 125 and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 187, 144 and 158, and

(15) an antibody containing heavy chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 123, 124 and 125 and light chain variable regions having CDRs 1, 2 and 3 consisting of the amino acid sequences set forth in SEQ ID NOs: 188, 144 and 158.

Among the antibodies described in (1) to (15), preferred is the antibody described in (15).

(IX) An antibody described in any of the following (1) to (15):

(1) an antibody having a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 191,

(2) an antibody having a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 192,

(3) an antibody having a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 193,

(4) an antibody having a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 194,

(5) an antibody having a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 195,

(6) an antibody having a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 196,

(7) an antibody having a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 197,

(8) an antibody having a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 198,

(9) an antibody having a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 199,

(10) an antibody having a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 200,

(11) an antibody having a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 201,

(12) an antibody having a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 202,

(13) an antibody having a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 203,

(14) an antibody having a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 204, and

(15) an antibody having a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 205.

Among the antibodies described in (1) to (15), preferred is the antibody described in (15).

(X) An antibody having a light chain variable region selected from the group consisting of the light chain variable regions described in the following (1) to (15):

(1) a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 191,

(2) a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 192,

(3) a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 193,

(4) a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 194,

(5) a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 195,

(6) a light chain variable region containing the amino acid

sequence set forth in SEQ ID NO: 196,

(7) a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 197,

(8) a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 198,

(9) a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 199,

(10) a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 200,

(11) a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 201,

(12) a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 202,

(13) a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 203,

(14) a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 204, and

(15) a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 205, and a heavy chain variable region selected from the group consisting of the heavy chain variable regions described in the following (1) to (7):

(1) a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 84,

(2) a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 85,

(3) a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 86,

(4) a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 87,

(5) a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 88,

(6) a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 89, and

Among the antibodies described above, preferred is the antibody having a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 205 and a heavy chain variable region containing the amino acid sequence set forth in SEQ ID NO: 90.

(XI) An antibody, in which one or more amino acids have been replaced, deleted, added and/or inserted in the amino acid sequence described in any one of the above-mentioned (I) to (X), and which has an activity equivalent to that of the antibody described in any of (I) to (X).

In the present invention, the activity equivalent to that of the antibody described in any of (I) to (X) means that the binding activity to a human glypican 3 antibody or the cytotoxicity activity on a cell that expresses human glypican 3 (e.g., HepG2 or a recombinant CHO cells expressing human glypican 3, etc.) is equivalent.

Humanized antibody

One preferred embodiment of the antibody according to the present invention is a humanized antibody that binds to glypican 3. The humanized antibody can be prepared by using a known method.

The humanized antibody is also referred to as a reshaped human antibody, which is made by transplanting the complementarity determining region (CDR) of an antibody of a non-human mammal, for example a mouse antibody, into the CDR of a human antibody. The general recombinant DNA technology for preparation of such antibodies is also known (see European Patent Application EP 125023 and International Patent Application WO 96/02576).

Specifically, for example, in the case where a CDR is derived from a mouse antibody, a DNA sequence which has been designed to link the CDRs of the mouse antibody with the framework region (FR) of a human antibody is synthesized by the PCR method using several oligonucleotides as primers, which have been prepared so as to have portions overlapping with one another at both ends of the CDR and the FR (see the method described in International Patent Application WO 98/13388).

As for the framework region of a human antibody to be linked with the CDR, the one which allows a complementarity determining region to form a favorable antigen-binding site is selected. If necessary, an amino acid in the framework

region of a variable region of the antibody may be replaced so that the complementarity determining region of a reshaped human antibody may form an appropriate antigen-binding site (Sato, K. et al., Cancer Res. (1993) 53, 851-856).

The C region of a human antibody may be used as the C region of a chimeric antibody or a humanized antibody, for example, Cγ1, Cγ2, Cγ3, and Cγ4 may be used in the H chain, and Cκ and Cλ may be used in the L chain. The C region of a human antibody may also be modified in order to improve the stability of the antibody or the production thereof. The human antibody to be used in the humanization may be any isotype of human antibody, for example, IgG, IgM, IgA, IgE and IgD, preferably, IgG, more preferably IgG1 or IgG3, and particularly preferably IgG1. the present invention IgG1 is effective when an antibody is used as an anticancer agent in terms of having a high cytotoxicity activity (Chemical immunology, 65: 88 (1997)).

In addition, after the humanized antibody is prepared, an amino acid in a variable region (e.g., FR) or a constant region may be replaced with another amino acid.

The origin of the CDR in a humanized antibody is not particularly limited, and the CDR may be derived from any animals. For example, it is possible to use a sequence derived from a mouse antibody, a rat antibody, a rabbit antibody, a camel antibody or the like. Preferred is a CDR sequence of a mouse antibody.

With regard to the humanization of an antibody, it is generally difficult to humanize it while maintaining the agonist activity of the original antibody. In the present invention, however, a humanized antibody having an agonist activity equivalent to that of the original mouse antibody was successful acquired. Since the antigenicity of the humanized antibody in the human body is reduced, it is useful in administering it into the human for a therapeutic purpose.

Preferred examples of the humanized anti- glypican 3 antibody in the present invention include, for example, an antibody having a heavy chain variable region set forth in SEQ ID NO: 84 (GC33 VH ver.a), SEQ ID NO: 85 (GC33 VH ver.c), SEQ ID NO: 86 (GC33 VH ver.f), SEQ ID NO: 87 (GC33 VH ver.h), SEQ ID NO: 88 (GC33 VH ver.i), SEQ ID NO: 89 (GC33 VH ver.j) or SEQ ID NO: 90 (GC33 VH ver.k) or an antibody having a light chain variable region set forth in SEQ ID NO: 92 (GC33 VL ver.a). Particularly preferred examples thereof include an antibody having a heavy chain variable region set forth in SEQ ID NO: 84 (GC33 VH ver.a), SEQ ID NO: 85 (GC33 VH ver.c), SEQ ID NO: 86 (GC33 VH ver.f), SEQ ID NO: 87 (GC33 VH ver.h), SEQ ID NO: 88 (GC33 VH ver.i), SEQ ID NO: 89 (GC33 VH ver.j) or SEQ ID NO: 90 (GC33 VH ver.k) and a light chain variable region set forth in SEQ ID NO: 92 (GC33 VL ver.a).

In addition, a preferred example of the humanized anti-glypican 3 antibody includes an antibody having a heavy

chain variable region containing the amino acid sequence set forth in SEQ ID NO: 90 and a light chain variable region containing the amino acid sequence set forth in SEQ ID NO: 205.

A preferred embodiment of the antibody according to the present invention is an antibody that binds to the epitope to which the antibody set forth in any of the following (1) to (8) binds:

(1) an antibody containing a heavy chain variable region having the amino acid sequence set forth in SEQ ID NO: 62 and a light chain variable region having the amino acid sequence set forth in SEQ ID NO: 73 (GC33),

(2) an antibody containing a heavy chain variable region having the amino acid sequence set forth in SEQ ID NO: 26 and a light chain variable region the amino acid sequence set forth in SEQ ID NO: 48 (M11F1),

(3) an antibody containing a heavy chain variable region having the amino acid sequence set forth in SEQ ID NO: 25 and a light chain variable region the amino acid sequence set forth in SEQ ID NO: 47 (M3B8),

(4) an antibody containing a heavy chain variable region having the amino acid sequence set forth in SEQ ID NO: 60 and a light chain variable region the amino acid sequence set forth in SEQ ID NO: 71 (GC199),

(5) an antibody containing a heavy chain variable region having the amino acid sequence set forth in SEQ ID NO: 61 and a light

chain variable region the amino acid sequence set forth in SEQ ID NO: 72 (GC202),

(6) an antibody containing a heavy chain variable region having the amino acid sequence set forth in SEQ ID NO: 63 and a light chain variable region the amino acid sequence set forth in SEQ ID NO: 74 (GC179),

(7) an antibody containing a heavy chain variable region having the amino acid sequence set forth in SEQ ID NO: 64 and a light chain variable region the amino acid sequence set forth in SEQ ID NO: 75 (GC194 (1)), and

(8) an antibody containing a heavy chain variable region having the amino acid sequence set forth in SEQ ID NO: 64 and a light chain variable region the amino acid sequence set forth in SEQ ID NO: 76 (GC194 (2)). More preferred is an antibody that binds to the epitope to which the antibody described in any of (1) to (5) binds, and particularly preferred is an antibody that binds to the epitope to which the antibody described in (1) binds.

The antibody that binds to the epitope to which any of the above-mentioned antibodies bind is useful because it has a particularly high cytotoxicity.

The antibody described in any of (1) to (7) binds to a region from the 524th amino acid to the 580th amino acid of human glypican 3. In particular, it binds to a region from the 524th amino acid to the 563rd amino acid. The antibody

described in any of (1) to (5) binds to a region from the 537th amino acid to the 563rd amino acid of human glypican 3. The antibody described in (1) binds to a region from the 544th amino acid to the 553rd amino acid of human glypican 3. In particular, it binds to a region from the 546th amino acid to the 551st amino acid.

The antibodies recognizing the above-mentioned epitopes have a high cytotoxicity, therefore they are useful in the treatment of a disease such as cancer. In particular, the antibody which binds to a region from the 546th amino acid to the 551st amino acid is useful as it has a particularly high cytotoxicity

Accordingly, the present invention includes the antibodies which binds to an epitope in a region from the 524th amino acid to the 580th amino acid of human glypican 3, preferably a region from the 524th amino acid to the 563rd amino acid, more preferably a region from the 537th amino acid to the 563rd amino acid, further more preferably a region from the 544th amino acid to the 553rd amino acid, particularly preferably a region from the 546th amino acid to the 551st amino acid.

Another preferred embodiment according to the present invention is an antibody that recognizes a region from the 524th amino acid to the 563rd amino acid of human glypican 3 and does not recognize a region from the 537th amino acid to the 563rd

amino acid.

A further preferred embodiment according to the present invention is an antibody that recognizes a region from the 537th amino acid to the 563rd amino acid of human glypican 3 and does not recognize a region from the 550th amino acid to the 563rd amino acid.

The analysis of an epitope recognized by an antibody can be carried out by a method known to those skilled in the art, for example, by Western blotting described in Examples below.

The antibody that recognizes the above-mentioned regions as an epitope can be obtained by a method known to those skilled in the art. For example, it can be obtained by preparing a peptide containing an amino acid sequence of a target region based on an amino acid sequence of human glypican 3 and preparing an antibody with the use of the peptide as an immunogene, or by preparing an antibody by a usual method and determining an epitope that the obtained antibody recognizes, and then selecting an antibody that recognizes the target epitope.

A preferred example of the anti-glypican 3 antibody of the present invention is an antibody having a high ADCC activity or an antibody having a high CDC activity to a cell that expresses glypican 3.

The phrase "a high ADCC activity" or "a high CDC activity" as used herein means that the antibody of the invention has

a higher ADCC activity or a higher CDC activity than that of a known anti-glypican 3 antibody. Known glypican 3 antibodies include, for example, M3C11 and M1E07 described in International Patent Application WO 2004/22739.

The ADCC activity or the CDC activity can be measured by a method known to those skilled in the art. For example, it can be measured by the chromium release test. Specific conditions of the chromium release test for measuring the ADCC activity are not particularly limited, however, for example, it can be measured using the conditions described in the Examples below.

Examples of the cells that express glypican 3 include, for example, a hepatoma cell line such as HepG2, a CHO cell line having a gene encoding glypican 3 incorporated therein and the like. To measure the ADCC activity, it is preferred to use a HepG2 cell line, and to measure the CDC activity, it is preferred to use a recombinant CHO cell line that expresses GPC3. The recombinant CHO cell line that expresses GPC3 may be prepared by any method, however, it can be prepared by, for example, the method described in the Examples below.

In the case where the anti-glypican 3 antibody is used as an anticancer agent, it is preferred that it has an ADCC activity at the same level as that of an antibody containing a heavy chain variable region having the amino acid sequence set forth in SEQ ID NO: 62 and a light chain variable region

having the amino acid sequence set forth in SEQ ID NO: 73 (GC33). In the case where the anti-glypican 3 antibody is used as an anticancer agent, it is preferred that it has a CDC activity at the same level as that of an antibody containing a heavy chain variable region having the amino acid sequence set forth in SEQ ID NO: 62 and a light chain variable region having the amino acid sequence set forth in SEQ ID NO: 73 (GC33).

Further, the present invention includes an antibody having a high binding activity to glypican 3.

In the present invention, the binding activity of the antibody to glypican 3 can be measured by using a method known to those skilled in the art. For example, it can be measured by utilizing the surface plasmon resonance with BIAcore. Specifically, a glypican 3 protein is immobilized on a sensor chip to react with an antibody, and the interaction between the antibody and glypican 3 can be calculated as a reaction rate constant from the measurement value. In addition, with regard to the evaluation of the binding activity, an enzyme linked immunosorbent assay (ELISA), an enzyme immunoassay (EIA), a radioimmunoassay (RIA) or a fluorescent antibody technique can be used. For example, in the case where an enzyme immunoassay is used, a sample containing an antibody to be tested, for example, a culture supernatant of a cell producing an antibody to be tested or a purified antibody is added to a plate which has been coated with an antigen to which the

antibody to be tested binds. Then, a secondary antibody labeled with an enzyme such as alkaline phosphatase is added, and the plated is incubated and washed. Then, an enzyme substrate such as p- nitrophenyl phosphate is added and the absorbance is measured, whereby an antigen binding activity can be evaluated. The upper limit of the binding activity is not particularly limited. However, for example, the upper limit can be defined within the range which is technically possible by those skilled in the art. It will be appreciated that the range which is technically possible will be expanded by the advancement of technology.

Further, in the present invention, an amino acid to be deamidated or an amino acid adjacent to an amino acid to be deamidated may be replaced with another amino acid for the purpose of, for example, suppressing deamidation to increase the stability of the antibody. The amino acid to be deamidated includes, asparagine and glutamine, preferably asparagine. An amino acid adjacent to asparagine is not particularly limited and may be any amino acid. It is known that an asparagine-glycine sequence is particularly susceptible to deamidation, thus, glycine is preferred as the amino acid adjacent to asparagine. An amino acid used for replacement is not particularly limited and may be any amino acid other than asparagine and glutamine. Preferred is an amino acid other than valine and proline. Therefore, in the present

invention, in the case where the antibody is deamidated, it is preferred to replace the amino acid with an amino acid other than asparagine, glutamine, valine and proline. Suppression of deamidation by amino acid replacement can be carried out with reference to, for example, International Patent Application WO 03/057881. In the case where amino acid replacement is carried out for the purpose of suppression of deamidation, it is preferred that the antigen binding activity before replacement is maintained.

Another embodiment of stabilization of the antibody includes replacement of glutamic acid with another amino acid. In addition, in the present invention, it was found that, in the case where the 6th amino acid of the heavy chain of an antibody is glutamic acid, the antibody can be significantly stabilized by replacing the glutamic acid with glutamine. Accordingly, the present invention also relates to a method of stabilizing an antibody by replacing the glutamic acid at the 6th position of the heavy chain of the antibody with glutamine. The amino acid numbering of the antibody is known to those skilled in the art (e.g., Kabat, E. A. et al., "Sequences of Proteins of Immunological Interest", US Dept. Health and Human Services 1983).

The antibody of the invention may be a conjugated antibody in which the antibody is conjugated with various molecules, such as polyethyleneglycol (PEG), radioactive

materials and toxin. Such a conjugated antibody may be prepared by chemically modifying the antibody obtained as above. Methods for modifying antibodies have already been established in the art. The antibody of the invention encompasses such a conjugated antibody.

The antibody of the invention may also be a bispecific antibody (see, for example, Journal of Immunology, 1994, 152, 5368-5374). The bispecific antibody may recognize glypican 3 and another antigen, or may recognize different epitopes on a GPC3 molecule.

Further, the antibody of the invention may carry a certain protein fused to the N- or C-terminus of the antibody (Clinical Cancer Research, 2004, 10, 1274-1281). The protein to be fused to the antibody may be conveniently selected by those skilled in the art.

In addition, the antibody of the invention includes an antibody with an enhanced cytotoxicity. Examples of the antibody with an enhanced cytotoxicity include an antibody lacking fucose, an antibody having bisecting N-acetyl glucosamine (GlcNAc) attached to its sugar chain, and an antibody having altered binding activity for Fcγ receptor obtained by substituting one or more amino acids in the Fc region. Such antibodies with an enhanced cytotoxicity can be prepared by a method known in the art.

Method of preparing antibody

The antibody that binds to glypican 3 can be prepared by a method known to those skilled in the art. For example, a monoclonal antibody-producing hybridoma can be prepared as follows basically using a known technique. That is, the hybridoma can be prepared by immunizing a mammal in accordance with a usual immunization method using a glypican 3 protein or a cell that expresses glypican 3 as a sensitizing antigen. The thus obtained immunocyte is fused with a known parent cell by a usual cell fusion method, and then selecting a monoclonal antibody-producing cell by a usual screening method.

Specifically, a monoclonal antibody can be prepared as follows. First, a glypican 3 protein is obtained based on the glypican 3 gene/amino acid sequence shown in SEQ ID NOs: 3 and 4, which is used as a sensitizing antigen to obtain an antibody. More specifically, the gene sequence encoding glypican 3 is inserted into a known expression vector system, and an appropriate host cell is transformed with the vector, and then a target human glypican 3 protein is purified by a known method from the host cell or the culture supernatant.

Subsequently, this purified glypican 3 protein is used as a sensitizing antigen. Alternatively, a partial peptide of glypican 3 can be used as a sensitizing antigen. In this case, the partial peptide can also be obtained by chemical synthesis according to the amino acid sequence of human

glypican 3.

The epitope on a glypican 3 molecule which is recognized by the anti-glypican 3 antibody of the present invention is not limited to a particular epitope. The anti-glypican 3 antibody may recognize any epitope, as long as the epitope is present on a glypican 3 molecule. Accordingly, any fragment can also be used as an antigen for producing the anti-glypican 3 antibody of the present invention, as long as it contains an epitope that is present on a glypican 3 molecule.

A mammal to be immunized with a sensitizing antigen is not particularly limited, but it is preferably selected in view of compatibility with a parent cell to be used for cell fusion. For example, rodents such as mice, rats and hamsters, rabbits or monkeys are generally used.

Immunization of an animal with a sensitizing antigen is carried out in accordance with a known method. For example, immunization is carried out by a general method in which a mammal is injected intraperitoneally or subcutaneously with a sensitizing antigen. Specifically, a sensitizing antigen is diluted with or suspended in an appropriate amount of PBS (Phosphate-Buffered Saline), physiological saline or the like, an appropriate amount of a standard adjuvant such as a Freund's complete adjuvant is mixed with the product if necessary, and then the solution is emulsified and is administered to a mammal several times every 4 to 21 days. In addition, an appropriate

carrier can also be used upon immunization with a sensitizing antigen.

A mammal is immunized as described above, and then an increased level of a target antibody in the serum is confirmed. Subsequently, immunocytes are collected from the mammal, and then subjected to cell fusion. A particularly preferred immunocyte is a splenocyte.

As a parent partner cell to be fused with the above-mentioned immunocyte, a mammalian myeloma cell is used. Examples of a cell line of the myeloma cell that is preferably used herein include various known cell lines such as P3 (P3x63 Ag8.653) (J. Immnol. (1979) 123, 1548-1550), P3x63 Ag8U.1 (Current Topics in Microbiology and Immunology (1978) 81, 1-7), NS-1 (Kohler. G. and Milstein, C. Eur. J. Immunol. (1976) 6, 511-519), MPC-11 (Margulies. D. H. et al., Cell (1976) 8, 405-415), SP2/0 (Shulman, M. et al., Nature (1978) 276, 269-270), FO (de St. Groth, S. F. et al., J. Immunol. Methods (1980) 35, 1-21), S194 (Trowbridge, I. S. J. Exp. Med. (1978) 148, 313-323) and R210 (Galfre, G. et al., Nature (1979) 277, 131-133).

Cell fusion of the above-mentioned immunocytes with myeloma cells can be basically carried out in accordance with a known method, for example, the method of Kohler and Milstein et al. (Kohler. G. and Milstein, C., Methods Enzymol. (1981) 73, 3-46).

More specifically, the above-mentioned cell fusion is carried out in a normal nutrition culture solution in the presence of, for example, a cell-fusion accelerator. As the cell-fusion accelerator, for example, polyethylene glycol (PEG), a hemagglutinating virus of Japan (HVJ) is used. If desired, an adjuvant such as dimethylsulfoxide can be added to further enhance the fusion efficiency.

The ratio of immunocytes to myeloma cells may be appropriately selected. For example, it is preferred that the number of immunocytes is 1 to 10 times greater than that of myeloma cells. The culture solution to be used for the above-mentioned cell fusion include, for example, a RPMI1640 culture solution or a MEM culture solution which is suitable for the growth of the above-mentioned myeloma cell line, or another normal culture solution which is used for this type of cell culture. Moreover, a serum supplement such as fetal calf serum (FCS) can be used in combination therewith.

Cell fusion is carried out as follows. Predetermined amounts of the above-mentioned immunocytes and myeloma cells are mixed well in the above-mentioned culture solution, a PEG (e.g., with an average molecular weight of approximately 1000 to 6000) solution (a general concentration of 30 to 60% (w/v)), which had been pre-heated at approximately 37°C, is added, and then the solution is mixed, whereby a target fusion cell (hybridoma) is formed. Subsequently, an appropriate culture

solution is added successively, and then a step of removing the supernatant by centrifugation is repeated to remove a reagent for cell fusion or the like that is unfavorable for the growth of the hybridoma.

The thus obtained hybridoma is selected by culturing the hybridoma in a standard selective culture solution such as a HAT culture solution (a culture solution containing hypoxanthine, aminopterin and thymidine). Cultivation in the above-mentioned HAT culture solution is continued for a time period sufficient for the cells (unfused cells) other than the target hybridoma to die (normally, several days to several weeks). Subsequently, a standard limiting dilution method is conducted to screen for and monoclone of hybridoma that produces a target antibody.

In addition to the method of immunizing a non-human animal with an antigen to obtain hybridoma, a desired human antibody having a binding activity to glypican 3 can also be obtained by sensitizing a human lymphocyte with glypican 3 in vitro, and allowing the sensitized lymphocyte to fuse with a human-derived myeloma cell having a permanent division potential (see JP-B-1-59878). In another method, glypican 3 antigen is administered to a transgenic animal having all the repertories of human antibody genes to obtain anti-glypican 3 antibody-producing cells, which are then immortalized, and a human antibody for glypican 3 may be obtained from the

immortalized anti-glypican 3 antibody-producing cells (see International Patent Applications WO 94/25585, WO 93/12227, WO 92/03918 and WO 94/02602).

The thus prepared hybridoma that produce a monoclonal antibody can be passage-cultured in a standard culture solution, or can be stored for a long period in liquid nitrogen.

One example of a method employed to obtain a monoclonal antibody from the hybridoma involves culturing the hybridoma and obtaining a monoclonal antibody from the culture supernatant in accordance with a standard method. Another method involves administering the hybridoma to a mammal that is compatible with the hybridoma to allow it to proliferate, and obtaining a monoclonal antibody from the ascites. The former method is suitable to obtain an antibody of high purity, while the latter method is suitable for the mass production of antibodies.

It is also possible to prepare a recombinant antibody by cloning the antibody gene from the hybridoma, incorporating the gene into an appropriate vector, introducing the vector into a host, and then allowing the host to produce the recombinant antibody by a genetic engineering technique (e.g., see Vandamme, A. M. et al., Eur. J. Biochem. (1990) 192, 767-775, 1990).

Specifically, mRNA encoding the variable (V) region of an anti-glypican 3 antibody is isolated from a hybridoma

producing the anti-glypican 3 antibody. mRNA is isolated by a known method such as a guanidine ultracentrifugal method (Chirgwin, J. M. et al., Biochemistry (1979) 18, 5294-5299) or an AGPC method (Chomczynski, P. et al., Anal. Biochem. (1987) 162, 156-159), and total RNA is prepared, and then target mRNA is prepared using an mRNA Purification Kit (Pharmacia) or the like. In addition, mRNA can also be directly prepared using a QuickPrep mRNA Purification Kit (Pharmacia).

The cDNA of the antibody V region is synthesized using a reverse transcriptase from the thus obtained mRNA. cDNA may be synthesized using an AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (SEIKAGAKU CORPORATION) or the like. In addition, for example, a 5'-Ampli FINDER RACE Kit (Clontech), the 5'-RACE method using PCR (Frohman, M. A. et al., Proc. Natl. Acad. Sci. USA (1988) 85, 8998-9002, Belyavsky, A. et al., Nucleic Acids Res. (1989) 17, 2919-2932) can be employed for synthesizing and amplifying cDNA.

A target DNA fragment is purified from the thus obtained PCR product, and then ligated to a vector DNA. A recombinant vector is prepared from the product, and then the vector is introduced into E. coli or the like, and a colony is selected, thereby preparing a desired recombinant vector. The nucleotide sequence of the target DNA is then determined by a known method such as a dideoxynucleotide chain termination method.

After DNA encoding the V region of the target anti-glypican 3 antibody is obtained, this DNA is incorporated into an expression vector containing DNA encoding the constant region (C region) of the target antibody.

To produce the anti-glypican 3 antibody used in the present invention, the antibody gene is incorporated into an expression vector so that the gene is expressed under the regulation of the gene expression control region including, for example, an enhancer and a promoter. Next, a host cell is transformed with the expression vector, thereby allowing the host to express the antibody.

An antibody gene can be expressed by incorporating a polynucleotide encoding the H chain or a polynucleotide encoding the L chain separately into an expression vector, and then simultaneously transforming a host cell with the vectors, or by incorporating polynucleotides encoding the H chain and the L chain into a single expression vector, and then transforming a host cell with the vector (see International Patent Application WO 94/11523).

Polynucleotide

In another aspect, the present invention provides a polynucleotide encoding a heavy chain variable region or a light chain variable region of the antibody of the present invention. Preferably, the polynucleotide of the present

invention has a nucleotide sequence described in any of SEQ ID NOs: 11-21, 33-43, 55-59, 65-70 and 77-83. In addition, a polynucleotide that is hybridized to the above-mentioned polynucleotide under stringent conditions and encodes an antibody having an activity equivalent to that of the antibody of the present invention is also within the scope of the present invention.

The polynucleotide of the present invention is not particularly limited as long as it encodes the antibody of the present invention. It is a polymer composed of a plurality of nucleotides, such as deoxyribonucleic acids (DNA) or ribonucleic acids (RNA). It may contain a base other than a naturally occurring base. The polynucleotide of the present invention can be used for producing an antibody by a genetic engineering technique. In addition, the polynucleotide of the present invention can be used as a probe to screen for an antibody having a function equivalent to that of the antibody of the present invention. That is, a polynucleotide encoding the antibody of the present invention or a partial fragment thereof may be used as a probe to obtain DNA that is hybridized to the polynucleotide under stringent conditions and encodes an antibody having an activity equivalent to that of the antibody of the present invention by techniques such as a hybridization technique, a gene amplification technique (e.g., PCR). Such DNA is also included in the polynucleotide of the

present invention.

The hybridization technique (Sambrook, J. et al., Molecular Cloning 2nd ed., 9.47-9.58, Cold Spring Harbor Lab. Press, 1989) is well known to those skilled in the art. Examples of the hybridization conditions include, for example, low stringent conditions. The low stringent conditions are, for example, the conditions of 42°C, 0.1x SSC and 0.1% SDS, preferably the conditions of 50°C, 0.1x SSC and 0.1% SDS when washing is performed after hybridization. More preferred examples of the hybridization conditions include, for example, high stringent conditions. The high stringent conditions are, for example, the conditions of 65°C, 5x SSC and 0.1% SDS. Under these conditions, it can be expected that a polynucleotide having a higher homology can be efficiently obtained under higher temperature. Incidentally, there are plural factors that affect the stringency of hybridization, such as temperature and the concentration of salt, and those skilled in the art can achieve a similar stringency by appropriately selecting these factors.

An antibody functionally equivalent to the antibody of the present invention encoded by a polynucleotide obtained by such a hybridization technique and a gene amplification technique usually has a high homology with the antibody in terms of the amino acid sequence. The antibody of the present invention also includes an antibody that is functionally

equivalent to the antibody of the present invention and has a high homology with the amino acid sequence of the antibody. A high homology means generally at least 50% or higher identity, preferably 75% or higher identity, more preferably 85% or higher identity, and further more preferably 95% or higher identity at the amino acid level. To determine the homology of polypeptides, the algorithm described in the literature (Wilbur, W. J. and Lipman, D. J., Proc. Natl. Acad. Sci. USA (1983) 80, 726-730) may be employed.

The present invention also provides a vector containing the polynucleotide of the present invention. Such a vector can be used for preparing the antibody of the present invention. As for the vector of the present invention, in the case where E. coli is used as a host, for example, it is not particularly limited as long as it has "ori" for use in amplification in E. coli to produce and amplify the vector in a large amount in E. coli (e.g., JM109, DH5α, HB101 or XL1Blue), and has a marker gene for selecting a transformed E. coli (e.g., a drug resistance gene that can be identified by a drug such as ampicillin, tetracycline, kanamycin or chloramphenicol). Examples of the vector include M13-series vectors, pUC-series vectors, pBR322, pBluescript, pCR-Script and the like. In addition, pGEM-T, pDIRECT, and pT7 can also be used for subcloning and extracting cDNA as well as the vectors described above.

As the vector of the present invention, an expression vector is particularly useful. For example, an expression vector to be expressed in E. coli should have the above characteristics to be amplified in E. coli. In addition, in the case where E. coli, such as JM109, DH5α, HB101, or XL1-Blue is used as a host cell, it is indispensable that the vector should have a promoter, for example, lacZ promoter (Ward et al., Nature (1989) 341, 544-546; FASEB J. (1992) 6, 2422-2427), araB promoter (Better et al., Science (1988) 240, 1041-1043), T7 promoter or the like, that can efficiently express the desired product in E. coli. Examples of such a vector include pGEX-5X-1 (Pharmacia), "QIAexpress system" (Qiagen), pEGFP, pET (in this case, the host is preferably BL21 which expresses T7 RNA polymerase) and the like, as well as the vectors described above.

In addition, the vector may also contain a signal sequence for polypeptide secretion. As for the signal sequence for protein secretion, in the case where a polypeptide is produced in the periplasm of E. coli, the pelB signal sequence (Lei S. P. et al., J. Bacteriol (1987) 169, 4379) can be used. Introduction of the vector into a host cell can be carried out by using, for example, the calcium chloride method and the electroporation method.

In addition to E. coli, for example, expression vectors derived from mammals (e.g., pcDNA3 (Invitrogen) and pEGF-BOS

(Nucleic Acids Res. (1990) 18(17), p5322), pEF and pCDM8), expression vectors derived from insect cells (e.g., "Bac-to-BAC baculovirus expression system" (GIBCO BRL) and pBacPAK8), expression vectors derived from plants (e.g., pMH1 and pMH2), expression vectors derived from animal viruses (e.g., pHSV, pMV and pAdexLcw), expression vectors derived from retroviruses (e.g., pZIPneo), expression vectors derived from yeast (e.g., "Pichia Expression Kit" (Invitrogen), pNV11 and SP-Q01), and expression vectors derived from Bacillus subtilis (e.g., pPL608 and pKTH50) can be used as the vector of the present invention.

For the purpose of expressing the vector in an animal cell such as a CHO cell, a COS cell, an NIH3T3 cell or the like, it is indispensable for the vector to have a promoter required for expression in a cell such as SV40 promoter (Mulligan et al., Nature (1979) 277, 108), MMTV-LTR promoter, EF1α promoter (Mizushima et al., Nucleic Acids Res. (1990) 18, 5322), CMV promoter or the like, and more preferably to have a marker gene (such as a drug resistance gene that can be identified by a drug such as neomycin or G418) for selecting transformation into the cell. Examples of the vector having such characteristics include, for example, pMAM, pDR2, pBK-RSV, pBK-CMV, pOPRSV and pOP13.

Further, for the purpose of stably expressing a gene and, at the same time, amplifying the gene copy numbers in the cell,

a vector (e.g., pCHOI, etc.) having the DHFR gene is introduced into the CHO cell deficient in the nucleic acid synthetic pathway to complement the deficiency and is amplified with methotrexate (MTX). In addition, for the purpose of transient expression of a gene, transformation is effected with a vector (such as pcD) having the origin of replication for SV40 using a COS cell having on the chromosome a gene that expresses the SV40 T antigen. As the origin of replication, the one derived from a polyoma virus, an adenovirus, a bovine papilloma virus (BPV) and the like can also be used. Further, for the amplification of gene copy numbers in the host cell system, the expression vector can include, as a selectable marker, the aminoglycoside transferase (APH) gene, the thymidine kinase (TK) gene, E. coli xanthine guaninephosphoribosyl transferase (Ecogpt) gene, the dihydrofolate reductase (dhfr) gene and the like.

To prepare the antibody of the present invention, the vector is introduced into a host cell. The host cell into which the vector is introduced is not particularly limited, but includes, for example, E. coli or any of various animal cells. For example, the host cell can be used as a production system for the production or expression of the antibody of the present invention. As for the production system of polypeptide preparation, there are an in vitro production system and an in vivo production system. In vitro production system include

a production system which employs eukaryotic cells and a production system which employs prokaryotic cells.

In the case where the eukaryotic cell is used, for example, an animal cell, a plant cell or a fungal cell can be used. Known animal cells include a mammalian cell such as a CHO cell (J. Exp. Med. (1995) 108, 945), a COS cell, a 3T3 cell, a myeloma cell, a baby hamster kidney (BHK) cell, a HeLa cell and a Vero cell, an amphibian cell such as a Xenopus oocyte (Valle, et al., Nature (1981) 291, 358-340), or an insect cell such as Sf9, Sf21, and Tn5. In the present invention, CHO-DG44, CHO-DXB11, a COS7 cell, a BHK cell are preferably used. Among the animal cells, for the purpose of performing a large amount of expression, a CHO cell is particularly preferred. Introduction of the vector into the host cell can be carried out by, for example, the calcium phosphate method, the DEAE-dextran method, the cationic ribozome DOTAP (Boehringer Mannheim), the electroporation method, the lipofection method or the like.

As for the plant cell, for example, a cell derived from Nicotiana tabacum is known as a protein production system, which may be subjected to callus culture. Examples of the fungal cells include yeast such as the genus Saccharomyces, more specifically Saccharomyces cerevistae and Saccharomyces pombe, and filamentous fungi such as the genus Aspergillus, more specifically Aspergillus niger.

In the case where the prokaryotic cell is used, production system using a bacterial cell may be employed. Examples of the bacterial cells include Escherichia coli (E. coli) such as JM109, DH5α and HB101, and Bacillus subtilis.

Preparation of recombinant antibody

The antibody of the present invention can be prepared by culturing the above-mentioned host cells. The antibody can be obtained by culturing in vitro a cell transformed with a desired polynucleotide. Cultivation can be carried out in accordance with a known method. Culture media for animal cells include, for example, DMEM, MEM, RPMI 1640, and IMDM. A serum supplement such as FBS or fetal calf serum (FCS) may be used in combination, or serum-free medium can be used. The pH during the cultivation is preferably about 6 to 8. Cultivation is usually carried out at about 30 to 40°C for about 15 to 200 hours with, as needed, medium change, aeration, and agitation.

On the other hand, systems for producing a polypeptide in vivo include, for example, a production system which employs an animal and a production system which employs a plant. The target polynucleotide is introduced into such an animal or a plant, and the polypeptide is produced in the body of the animal or the plant and recovered. The term "host cell" as used herein encompasses such an animal and a plant.

When the animal is used, production systems employing

a mammal or an insect are available. As the mammal, goats, pigs, sheep, mice and cattle can be used (Vicki Glaser, SPECTRUM Biotechnology Applications, 1993). A transgenic animal can also be used as a mammal.

For example, the target polynucleotide is prepared as a fusion gene with a gene encoding a polypeptide which is inherently produced in the milk such as goat β casein. Then, the DNA fragment containing this fusion gene is injected into a goat embryo, and the embryo is transplanted into a female goat. The target antibody can be obtained from the milk produced by the transgenic goat borne to the goat which received the embryo or the offspring thereof. To increase the amount of milk containing the antibody produced by the transgenic goat, hormone may be given to the transgenic goat as needed. (Ebert, K. M. et al., Bio/Technology (1994) 12, 699-702).

In addition, as an insect, for example, a silkworm can be used. In the case where a silkworm is used, a silkworm is infected with a baculovirus into which the polynucleotide encoding the target antibody has been inserted. The target antibody can be obtained from the body fluid of the silkworm (Susumu, M. et al., Nature (1985) 315, 592-594).

In the case where a plant is used, for example, tobacco can be used. In the case where tobacco is used, a polynucleotide encoding the target antibody is inserted into an expression vector for a plant, for example pMON 530, and

then the vector is introduced into a bacterium such as Agrobacterium tumefaciens. Then, tobacco such as Nicotiana tabacum is infected with the bacterium, whereby the target antibody can be obtained from the leaves of the tobacco (Julian, K. -C. Ma et al., Eur. J. Immunol. (1994) 24, 131-138).

The thus obtained antibody can be isolated from the inside or the outside (culture medium, etc.) of the host cell and then can be purified to a substantially pure and uniform antibody. Separation and purification of the antibody may be carried out by a separation and a purification method usually used in purification of polypeptides. For example, polypeptides can be separated and purified by any methods including chromatography columns, filtration, ultrafiltration, salting-out, solvent precipitation, solvent extraction, distillation, immunoprecipitation, SDS-polyacrylamide gel electrophoresis, isoelectric focusing, dialysis, recrystallization, and a combination thereof.

Examples of the chromatography include, for example, affinity chromatography, ion exchange chromatography, hydrophobic chromatography, gel-filtration, reverse phase chromatography, adsorption chromatography (Strategies for Protein Purification and Characterization: A Laboratory Course Manual. Ed Daniel R. Marshak et al., Cold Spring Harbor Laboratory Press, 1996). These chromatographies can be carried out using a liquid phase chromatography such as HPLC

and FPLC. Examples of a column to be used for affinity chromatography include a protein A column or a protein G column. One example of the protein A column is Hyper D, POROS, Sepharose F. F. (Pharmacia).

Further, before or after purification of the antibody, the antibody can be modified or peptides can be partially removed as needed by allowing a suitable protein-modifying enzyme to act on. The protein-modifying enzyme for this purpose include, for example, trypsin, chymotrypsin, lysyl endopeptidase, protein kinase, glucosidase.

Diagnostic method

In another aspect, the present invention provides a method of diagnosing a disease such as cancer by detecting GPC3 protein in a test sample with the use of the antibody of the present invention.

The detection used herein includes quantitative detection and non-quantitative detection. The non-quantitative detection include, for example, determination of merely whether or not GPC3 protein is present, determination of whether or not a specific amount or more of GPC3 protein is present, determination for comparison of the amount of GPC3 protein with that of another sample (e.g., a control sample). The quantitative detection includes determination of the concentration of GPC3 protein, determination of the amount of

GPC3 protein.

The test sample is not particularly limited as long as it is a sample that may possible contain GPC3 protein, however, preferred is a sample collected from the body of a living organism such as a mammal, and more preferred is a sample collected from human. Specific examples of the test sample may include, for example, blood, interstitial fluid, plasma, extravascular fluid, cerebral fluid, joint fluid, pleural fluid, serum, lymph fluid, saliva, preferably blood, serum and plasma. In addition, a sample obtained from the test sample such as culture solution of cells collected from the body of the living organism is also included in the test sample of the present invention.

The cancer to be diagnosed is not particularly limited, and specific examples may include liver cancer, pancreatic cancer, lung cancer, colon cancer, mammary cancer, prostate cancer, leukemia and lymphoma, preferably liver cancer. GPC3 to be detected is not particularly limited, and may be either full-length GPC3 or a fragment thereof. In the case where a fragment of GPC3 is detected, it may be either the N-terminal fragment or the C-terminal fragment, however, the N-terminal fragment is preferred. In addition, the GPC3 protein may also be a heparan sulfate-added GPC3 or a GPC3 core protein.

The method of detecting GPC3 protein contained in a test sample is not particularly limited, however, detection is

preferably performed by an immunological method with the use of an anti-GPC3 antibody. Examples of the immunological method include, for example, a radioimmunoassay, an enzyme immunoassay, a fluorescence immunoassay, a luminescence immunoassay, immunoprecipitation, a turbidimetric immunoassay. Preferred is an enzyme immunoassay, and particularly preferred is an enzyme-linked immunosorbent assay (ELISA) (e.g., a sandwich ELISA). The above-mentioned immunological method such as an ELISA can be carried out by a method known to those skilled in the art.

A general detection method with the use of an anti-GPC3 antibody comprises immobilizing an anti-GPC3 antibody on a support, adding a test sample thereto, incubating the support to allow the anti-GPC3 antibody and GPC3 protein to bind to each other, washing the support, and detecting the GPC3 protein binding to the support via the anti-GPC3 antibody to detect GPC3 protein in a test sample.

The binding between the anti-GPC3 antibody and the GPC3 protein is generally carried out in a buffer. Buffers used in the invention include, for example, a phosphate buffer, a Tris buffer. Incubation is carried out under the conditions generally employed, for example, at 4°C to room temperature for 1 hour to 24 hours. The washing after incubation can be carried out by any method as long as it does not inhibit the binding between the GPC3 protein and the anti-GPC3 antibody,

using for example a buffer containing a surfactant such as Tween 20.

In the method of detecting GPC3 protein of the present invention, a control sample may be provided in addition to a test sample to be tested for GPC3 protein. The control samples include a negative control sample that does not contain GPC3 protein and a positive control sample that contains GPC3 protein. In this case, it is possible to detect GPC3 protein in the test sample by comparing the result obtained with the negative control sample that does not contain GPC3 protein with the result obtained with the positive control sample that contains GPC3 protein. It is also possible to quantitatively detect GPC3 protein contained in the test sample by obtaining the detection results of the control samples and the test sample as numerical values, and comparing these numerical values.

One preferred embodiment of detecting GPC3 protein binding to the support via an anti-GPC3 antibody is a method using an anti-GPC3 antibody labeled with a detectable label. For example, GPC3 protein may be detected by contacting the test sample with an anti-GPC3 antibody immobilized on the support, washing the support, and then detecting GPC3 with the use of the labeled antibody that specifically binds to GPC3 protein.

The labeling of an anti-GPC3 antibody can be carried out by a generally known method. Examples of the detectable label

known to those skilled in the art include a fluorescent dye, an enzyme, a coenzyme, a chemiluminescent substance or a radioactive substance. Specific examples may include radioisotopes (^{32}P, ^{14}C, ^{125}I, ^{3}H, ^{131}I and the like), fluorescein, rhodamine, dansyl chloride, umbelliferone, luciferase, peroxidase, alkaline phosphatase, β-galactosidase, β-glucosidase, horseradish peroxidase, glucoamylase, lysozyme, saccharide oxidase, microperoxidase, biotin and the like. In the case where biotin is used as a detectable label, it is preferred that a biotin-labeled antibody is added, and then avidin conjugated to an enzyme such as alkaline phosphatase is further added.

Specifically, a solution containing an anti-GPC3 antibody is added to a support such as a plate to allow the anti-GPC3 antibody to be immobilized. After washing, the plate is blocked with, for example, BSA in order to prevent the nonspecific binding of a protein. The plate is washed again, and then the test sample is added to the plate. After being incubated, the plate is washed, and then the labeled anti-GPC3 antibody is added. After being incubated appropriately, the plate is washed, and then the labeled anti-GPC3 antibody remaining on the plate is detected. The detection of the protein can be carried out by a method known to those skilled in the art. For example, in the case where the antibody is labeled with a radioactive substance, the protein may be

detected by liquid scintillation or the RIA method. In the case where the antibody is labeled with an enzyme, the protein may be detected by adding a substrate and detecting an enzymatic change of the substrate such as color development with an absorbance reader. In the case where the antibody is labeled with a fluorescent substance, the protein may be detected with the use of a fluorometer.

A particularly preferred embodiment of the method of detecting GPC3 protein of the present invention is a method using an anti-GPC3 antibody labeled with biotin and avidin. Specifically, a solution containing an anti-GPC3 antibody is added to a support such as a plate to allow the anti-GPC3 antibody to be immobilized thereon. After washing, the plate is blocked with, for example, BSA in order to prevent the nonspecific binding of a protein, The plate is washed again, and then the test sample is added to the plate. After being incubated, the plate is washed, and then the biotin-labeled anti-GPC3 antibody is added. After being incubated appropriately, the plate is washed, and then avidin conjugated to an enzyme such as alkaline phosphatase or peroxidase is added. After being incubated, the plate is washed, and then a substrate of the enzyme conjugated to avidin is added. Then, GPC3 protein is detected by means of the enzymatic change of the substrate as an indicator.

Another embodiment of the method of detecting GPC3

protein of the present invention is a method using a primary antibody that specifically binds to GPC3 protein and a secondary antibody that specifically binds to the primary antibody. For example, the test sample is brought into contact with an anti-GPC3 antibody immobilized on the support, the support is incubated and washed, and the bound GPC3 protein after washing is detected with a primary anti-GPC3 antibody and a secondary antibody that specifically binds to the primary antibody. In this case, the secondary antibody is preferably labeled with a detectable label.

Specifically, a solution containing an anti-GPC3 antibody is added to a support such as a plate to allow the anti-GPC3 antibody to be immobilized thereon. After washing, the plate is blocked with, for example, BSA in order to prevent the nonspecific binding of a protein. The plate is washed again, and then the test sample is added to the plate. After being incubated, the plate is washed, and then a primary anti-GPC3 antibody is added. After being incubated appropriately, the plate is washed, and then a secondary antibody that specifically binds to the primary antibody is added. After being incubated appropriately, the plate is washed, and then the secondary antibody remaining on the plate is detected. The detection of the secondary antibody can be carried out by the above-mentioned method.

Pharmaceutical composition

In another aspect, the present invention provides a pharmaceutical composition containing the antibody of the present invention. The pharmaceutical composition containing the antibody of the present invention is useful in the treatment and/or prevention of a disease associated with cell proliferation such as cancer, and particularly it is useful in the treatment and/or prevention of liver cancer. In the case where the antibody of the present invention is used as a pharmaceutical composition, the antibody can be formulated into a dosage form by a method known to those skilled in the art. For example, the pharmaceutical composition can be used parenterally in the form of an injection of a sterile solution or a suspension with water or another pharmaceutically acceptable solution. For example, the antibody can be formulated into a dosage form by appropriately mixing it with a pharmaceutically acceptable carrier or solvent, such as sterile water, physiological saline, a plant-oil, an emulsifier, a suspension, a surfactant, a stabilizer, a flavor, an excipient, a vehicle, a preservative, a binder to prepare a unit dosage form required for generally accepted Drug Implementation. The amount of active ingredients in these preparations is selected to allow for administration of a suitable dosage within the indicated range.

A sterile composition for injection can be formulated

by using a vehicle such as distilled water for injection in accordance with the general Drug Implementation.

Examples of the aqueous solution for injection include, for example, physiological saline, glucose, and other isotonic liquids including adjuvants, such as D-sorbitol, D-mannnose, D-mannitol and sodium chloride. They can be used in combination with a suitable solubilizer, such as an alcohol, specifically ethanol, a polyalcohol such as propylene glycol and polyethylene glycol, and a non-ionic surfactant such as Polysorbate 80 (TM) and HCO-50.

Sesame oil or soybean oil can be used as a oleaginous liquid and may be used in combination with benzyl benzoate or benzyl alcohol as a solubilizer. It may be formulated with a buffer such as a phosphate buffer or a sodium acetate buffer, a pain-killer such as procaine hydrochloride, a stabilizer such as benzyl alcohol or phenol, or an antioxidant. The prepared injection is generally filled into a suitable ampule.

The method of administration is preferably parenteral, and specific examples thereof include injection, transnasal administration, transpulmonary administration, transdermal administration and the like. The injection formulation may be administered systemically or topically by intravenous injection, intramuscular injection, intraperitoneal injection, subcutaneous injection or the like.

The method of administration can be appropriately

selected according to the age and the symptoms of a patient. For example, one dose of the pharmaceutical composition containing the antibody or the polynucleotide encoding the antibody can be selected from the range of 0.0001 mg to 1,000 mg per kg of body weight. Alternatively, for example, the dose can be selected from the range of 0.001 mg to 100,000 mg/body per patient, although it is not always limited to these numerical values. The dose and the method of administration vary according to the body weight, the age and the symptoms of a patient, and are appropriately selected by those skilled in the art.

All patents and references cited in this specification are incorporated by reference. All the contents disclosed in the specifications and drawings of Japanese Patent Application No. 2004-203637, on which the application claims priority, are incorporated herein by reference.

EXAMPLE

The present invention will be described in more detail with reference to Examples below. However, the present invention is not limited to these Examples.

Example 1

cDNA cloning of human glypican 3 (GPC3)

A full-length cDNA encoding human GPC3 was amplified by

PCR reaction with an Advantage 2 kit (CLONTECH) using 1st stranded cDNA prepared by a usual method from a colon cancer cell line, Caco2, as a template. More specifically, 50 μL of a reaction mixture containing 2 μL of cDNA derived from Caco2, 1 μL of a sense primer (GATATC-ATGGCCGGGACCGTGCGCACCGCGT: SEQ ID NO: 1), 1 μL of an antisense primer (GCTAGC-TCAGTGCACCAGGAAGAAGAAGCAC: SEQ ID NO: 2), 5 μL of Advantage 2 10x PCR buffer, 8 μL of dNTP mix (1.25 mM) and 1.0 μL of Advantage polymerase Mix was subjected to 35 cycles consisting of 94°C for 1 minute, 63°C for 30 seconds and 68°C for 3 minutes. The amplified product from the PCR reaction was inserted into a TA vector, pGEM-T Easy, using pGEM-T Easy Vector System I (Promega). The sequence was confirmed by using an ABI 3100 DNA sequencer. In this way, a cDNA encoding full-length human GPC3 was isolated. The sequence shown in SEQ ID NO: 3 indicates the nucleotide sequence of human GPC3 gene and the sequence shown in SEQ ID NO: 4 indicates the amino acid sequence of human GPC3 protein.

Example 2

Preparation of soluble form of GPC3

As immunoprotein for the generation of an anti-GPC3 antibody, a soluble form of GPC protein was prepared, in which a hydrophobic region at the C-terminal side (564-580 amino acids) was deleted.

By using the full-length human GPC3 cDNA as a template, a PCR reaction was carried out using an antisense primer (ATA GAA TTC CAC CAT GGC CGG GAC CGT GCG C: SEQ ID NO: 5) and a sense primer, to which an EcoRI recognition sequence and a Kozak sequence were added, (ATA GGA TCC CTT CAG CGG GGA ATG AAC GTT C: SEQ ID NO: 6). The obtained PCR fragment (1711 bp) was cloned into pCXND2-Flag. The pCXND2-Flag was designed to express a Flag-tagged protein by inserting the region for DHFR gene expression of pCHOI (Hirata et al., FEBS letter 1994; 356; 244-248) into the HindIII site of pCXN2 (Niwa et al., Gene 1991; 108; 193-199) and adding a Flag tag sequence to the downstream of the multicloning site. The constructed expression plasmid DNA was introduced into a CHO cell line, DXB11, and a CHO cell line highly expressing the soluble form of GPC3 was obtained by selection with 500 μg/mL Geneticin. The large-scale cultivation of the CHO cell line highly expressing the soluble form of GPC3 was carried out using a 1700-cm^2 roller bottle, and the culture supernatant was recovered for the antibody purification. The culture supernatant was applied to a DEAE sepharose Fast Flow column (Amersham) and, after washing, the antibody was eluted with a buffer containing 500 mM NaCl, and affinity purified using Anti-Flag M2 agarose affinity gel (SIGMA). The elution was carried out with 200 μg/mL FLAG peptide. After the eluate was concentrated with Centriprep-10 (Millipore), FLAG peptide was removed by gel filtration using

Superdex 200 HR 10/30 (Amersham). Lastly, the filtrate was concentrated using a DEAE sepharose Fast Flow column and eluted with PBS (containing 500 mM NaCl) without Tween 20 to effect buffer exchange.

Example 3

Preparation of soluble form of GPC3 core protein

GPC3 is modified by heparan sulfate to become a macromolecule. To eliminate an antibody against heparan sulfate in a screening for an anti-GPC3 antibody, a soluble form of GPC3 core protein that had a point mutation in the heparan sulfate-binding site was prepared and used in the screening.

Using the above-mentioned soluble form of GPC3 (1-563) as a template, a cDNA in which Ser residues at the 495th and 509th positions were replaced with Ala was prepared by the assembly PCR method, in which primers were designed to add His tag to the C-terminus. The obtained cDNA was cloned into pCXND3 vector. The pCXND3 was constructed by inserting the DHFR gene expressing region of pCHOI in the HindIII site of pCXN2. The constructed expression plasmid DNA was introduced into DXB11 cell line and a CHO cell line highly expressing a soluble form of GPC3 core protein was obtained by selection with 500 μg/mL Geneticin.

The large-scale cultivation was carried out using a

1700-cm^2 roller bottle and the culture supernatant was recovered for antibody purification. The culture supernatant was applied to a Q sepharose Fast Flow column (Amersham). After washing, the antibody was eluted with a phosphate buffer containing 500 mM NaCl, and affinity purified using a Chelating sepharose Fast Flow column (Amersham). The antibody was eluted with a gradient of 10 to 150 mM imidazole. Lastly, the eluate was concentrated using a Q sepharose Fast Flow column and, eluted with a phosphate buffer containing 500 mM NaCl.

SDS polyacrylamide gel electrophoresis under reducing conditions showed three bands of 70 kDa, 40 kDa and 30 kDa. The result of amino acid sequencing using an ABI492 protein sequencer (Applied Biosystems) indicated that the 30 kDa band corresponded to the amino acid sequence of the 359th and its downstream or the 375th and its downstream of GPC3, suggesting that GPC3 was cleaved between Arg358 and Ser359 or between Lys374 and Val375, hence, it was separated into 40 kDa of the N-terminal fragment and 30 kDa of the C-terminal fragment.

Example 4

Preparation of CHO cell line expressing full-length human GPC3

To obtain a cell line for evaluating a binding activity using flow cytometry, a CHO cell line expressing full-length GPC3 was established.

Ten microgram of a full-length human GPC3 gene expression

vector and 60 μL of SuperFect (QIAGEN) were mixed. After a complex was formed, gene introduction was carried out by adding it to a CHO cell line, DXB11. After a 24-hour cultivation in a CO_2 incubator, selection was started using αMEM (GIBCO BRL) containing Geneticin at a final concentration of 0.5 mg/mL and 10% FBS. The resulting Geneticin-resistant colonies were collected and cell cloning was carried out by the limiting dilution method. Each cell clone was solubilized and the expression of full-length human GPC3 was confirmed by Western blotting using an anti-GPC3 antibody. In this way, a stably expressing cell line was obtained.

Example 5

Evaluation of binding activity by ELISA

The soluble form of GPC3 core protein was diluted to 1 μg/mL with a coating buffer (0.1 mol/L $NaHCO_3$ (pH 9.6), 0.02% (w/v) NaN_3) and added to an immunoplate and left at 4°C overnight to coat the plate. After the plate was blocked with a dilution buffer (50 mmol/L Tris-HCl (pH 8.1), 1 mmol/L $MgCl_2$, 150 mmol/L NaCl, 0.05% (v/v) Tween 20, 0.02% (w/v) NaN_3, 1% (w/v) BSA), an anti-GPC3 antibody was added and left at room temperature for 1 hour. After washing with a rinse buffer (0.05% (v/v) Tween 20, PBS), an anti-mouse IgG antibody (ZYMED) labeled with alkaline phosphatase was added and left at room temperature for 1 hour. After washing with the rinse buffer, SIGMA 104

(SIGMA) diluted to 1 mg/mL with a substrate buffer (50 mmol/L $NaHCO_3$ (pH 9.8), 10 mmol/L $MgCl_2$) was added and left at room temperature for 1 hour for color development. Then the absorbance (at 405 nm, reference wavelength of 655 nm) was measured using a Benchmark Plus (BIO-RAD).

Example 6

Immunization with soluble form of GPC3 and selection of hybridoma

Since human GPC3 and mouse GPC3 show a high homology of 94% at the amino acid level, it was considered difficult to obtain an anti-GPC3 antibody if a normal mouse was immunized. Therefore, an autoimmune disease mouse, MRL/MpJUmmCrj-lpr/lpr mouse, (hereinafter referred to as MRL/lpr mouse, purchased from Charles River Japan, Inc.) was used as an immunized animal. Immunization was started at the age of 7 weeks or 8 weeks. For the first immunization, a soluble form of GPC3 was prepared at 100 μg/head and emulsified using Freund's complete adjuvant (FCA, Becton Dickinson) and subcutaneously administered. Two weeks later, a soluble form of GPC3 was prepared at 50 μg/head and emulsified using Freund's incomplete adjuvant (FIA, Becton Dickinson) and subcutaneously administered. After that, an additional immunization was carried out every other week for 5 times in total. To two of the immunized mice, a soluble form of GPC3 was diluted with PBS to 50 μg/head, and then

administered intravenously via the tail as the final immunization. On the forth day after the final immunization, the spleen was excised to obtain a spleen cell, which was mixed with a mouse myeloma cell, P3-X63Ag8U1 (P3U1, purchased from ATCC), at a ratio of 2:1. Cell fusion was carried out by gradually adding PEG 1500 (Roche Diagnostic). RPMI 1640 medium (GIBCO BRL) was carefully added to dilute PEG 1500, and after PEG 1500 was removed by centrifugation, the cells were suspended in RPMI 1640 medium containing 10% FBS and inoculated into a 96-well culture plate at 100 μL/well. On the next day, RPMI 1640 medium containing 10% FBS, 1x HAT media supplement (SIGMA) and 0.5x BM-Condimed H1 Hybridoma cloning supplement (Roche Diagnostic) (hereinafter referred to as HAT medium) was added at 100 μL/well. After 2, 3 and 5 days, half of the culture solution was replaced with the HAT medium. After 7 days, screening was carried out using the culture supernatant. The screening was carried out by an ELISA using an immunoplate coated with the soluble form of GPC3 core protein. A positive clone was monocloned by the limiting dilution method. As a result, 11 clones of antibodies (M3C11, M13B3, M1E7, M3B8, M11F1, L9G11, M19B11, M6B1, M18D4, M5B9 and M10D2) that have a strong binding activity against GPC3 were obtained.

Example 7

Isotype determination and purification of anti-GPC3 antibody

Isotype was determined by an antigen-dependent ELISA using an Immunopure Monoclonal Antibody Isotyping Kit I (PIERCE). The purification of antibodies was carried out as follows. The culture supernatant of hybridoma cultured with the HAT medium supplemented with FBS (Ultra low IgG) (GIBCO BRL) was adsorbed to Hi Trap ProteinG HP (Amersham), and washed with a binding buffer (20 mM sodium phosphate (pH 7.0)). The antibody was eluted with an elution buffer (0.1 M glycine-HCl (pH 2.7)). The eluate was immediately neutralized with a neutralization buffer (1 M Tris-HCl (pH 9.0)), and dialyzed against PBS for day and night for buffer exchange.

Example 8

Evaluation of binding activity by ELISA

In order to conveniently evaluate the binding activity of the anti-GPC3 antibody thus obtained, concentration-dependent binding of the antibody was detected against an immunoplate containing the soluble form of GPC3 core protein immobilized thereon. A 3-fold dilution series (12 dilutions in total) of the purified antibody at a concentration of 10 μg/mL was added, and an anti-mouse IgG antibody was added as the secondary antibody. Color development was carried out using SIGMA 104. Since the degree of color development varies depending on the color development time, data measured precisely after 1 hour was analyzed. Every antibody showed

a concentration-dependent color development. The correlation between the concentration of antibody and the degree of color development was plotted and an approximate curve was obtained by using an analyzing software, GraphPad Prism. Its EC50 value was determined as the index of the binding activity. EC50 values for all clones are shown in Fig. 16.

Example 9

Evaluation of binding activity by flow cytometry

Cells were dissociated with 1 mM EDTA pH 8.0 (GIBCO)/PBS and suspended in FACS buffer (1% FBS/PBS) at 1 x 10^6 cells/mL. The suspension was dispensed to a Multiscreen-HV Filter Plate (Millopre) at 100 μL/well and the supernatant was removed by centrifugation. An anti-GPC3 antibody diluted to an appropriate concentration was added and reacted on ice for 30 minutes. The cells were washed once with FACS buffer and an FITC-labeled anti-mouse IgG antibody was added and reacted on ice for 30 minutes. After the reaction, the cells were centrifuged at 500 rpm for 1 minute, and the supernatant was removed. The cells were suspended in 400 μL of FACS buffer and subjected to flow cytometry. EPICS ELITE ESP (Beckman Coulter) was used as a flow cytometer. A gate was set on the living cell population with the histogram of forward scatter and side scatter. As shown in Fig. 1, an anti-GPC3 antibody (M3C11, M11F1) bound strongly to the CHO cell expressing GPC3

and did not bind to the parent CHO cell, indicating that the antibody specifically binds to GPC3 presented on the cell membrane. In addition, the antibody showed the binding activity to a hepatoma cell line, HepG2 (purchased from ATCC) and HuH-7 (purchased from Health Science Research Resources Bank), suggesting that the antibody may specifically recognize hepatoma. The binding activity of the clones derived from the mouse immunized with a soluble form of GPC3 measured by flow cytometry is shown in Fig. 16, where the X-mode values of histogram at the concentration of antibody of 5 μg/mL are indicated.

Example 10

Epitope classification by competitive ELISA

The obtained antibodies were classified according to the epitopes by a competitive ELISA. The antibodies were biotinylated using a Biotin Labeling Kit (Roche). The soluble form of GPC3 core protein was diluted to 1 μg/mL with the coating buffer and added to a plate at 100 μL/well and stored at 4°C overnight to coat the plate. On the next day, 200 μL of the substrate buffer was added for blocking. The plate was left at 4°C overnight or longer and an anti-GPC3 antibody was added to the plate at 100 μL/well and reacted at room temperature for 1 hour. After that, without washing of the plate, 10 μL of 10 μg/mL biotin-labeled anti-GPC3 antibody was added and

further reacted for 1 hour. The plate was washed with 300 μL/well of the rinse buffer for 3 times. AP-streptavidin conjugate (ZYMED) was diluted to 1000-fold with the dilution buffer and added at 100 μL/well and reacted at room temperature for 1 hour. The plate was washed with 300 μL/well of the rinse buffer for 5 times. SIGMA 104 was diluted to 1 mg/mL with the substrate buffer and added at 100 μL/well. After incubating for 1 hour at room temperature, the absorbance (at 405 nm, reference wavelength of 655 nm) was measured.

The results of the competitive ELISA are shown in Fig. 2. As for the antibody that competitively inhibited the binding of the biotinylated antibody by 50% or more, it was considered that its epitopes are located close together in the three-dimensional conformation. As a result of classification according to the competitive inhibition pattern of color development against the binding of the 8 types of biotinylated antibodies, the 11 clones derived from the mouse immunized with a soluble form of GPC3 were classified into 5 groups (a, b, c, d and e) (Fig. 16).

Example 11

Epitope classification by Western blotting

The soluble form of GPC3 core protein was applied to a 10% SDS-PAGE mini (TEFCO) and electrophoresed under reducing conditions. It was transferred to Immobilon-P (Millipore)

using Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (BIO-RAD). After the membrane was briefly washed with TBS-T (0.05% Tween 20, TBS), it was shaken in TBS-T containing 5% skim milk for 1 hour. The membrane was shaken in TBS-T for about 10 minutes, then each anti-GPC3 antibody diluted with TBS-T containing 1% skim milk was added and the membrane was shaken for 1 hour. The membrane was washed with TBS-T and shaken in a solution of HRP-anti-mouse IgG antibody (Amersham) diluted with TBS-T containing 1% skim milk for 1 hour, and then washed with TBS-T. Color development was carried out using ECL-Plus (Amersham) and detected using Hyperfilm ECL (Amersham).

As shown in Fig. 3, L9G11 was determined to be an antibody binding to the N-terminal side because it bound to the band of about 40 kDa. M3C11 was determined to be an antibody binding to the C-terminal side because it bound to the band of about 30 kDa. All the antibodies belonging to c, d or e group based on the competitive ELISA bound to the N-terminal side, and all those belonging to a or b groups bound to the C-terminal side (Fig. 16). L9G11 had higher detection sensitivity in Western blotting than the other antibodies that bind to the N-terminal side, suggesting that this antibody is a useful for detecting the N-terminal fragment by Western blotting.

Example 12

Detection of secreted form of GPC3

Since it was found that GPC3 is cleaved at the 358th amino acid residue or the 374th amino acid residue, the inventors hypothesized that a secreted form of GPC3 is secreted into the blood of a patient with liver cancer. Therefore, a GPC3 sandwich ELISA system was constructed in order to detect a secretory form of GPC3.

An immunoplate was coated with an anti-GPC3 antibody at 10 μg/mL and blocked by the substrate buffer. After the immunoplate was stored for several hours at room temperature or overnight at 4°C, the culture supernatant of HepG2 was added and incubated for 1 hour at room temperature. The immunoplate was washed with 300 μL/well of the rinse buffer for 3 times, and a biotin-labeled anti-GPC3 antibody diluted to 10 μg/mL was added and incubated for 1 hour at room temperature. The immunoplate was washed with 300 μL/well of the rinse buffer for 3 times, and AP-streptavidin was added and incubated for 1 hour at room temperature. The immunoplate was washed with 300 μL/well of the rinse buffer for 5 times. Color development was carried out using AMPAK (DAKO) in accordance with the attached protocol and the absorbance was measured using a microplate reader. The antibodies binding to the N-terminal side (M6B1, M19B11 and M18D4) and those binding to the C-terminal side (M3C11, M13B3 and M3B8) were combined to construct five sandwich ELISA systems. Each of these

combinations showed an equivalent sensitivity in the standard curve using the secreted form of GPC3. These systems were evaluated using the culture supernatant of HepG2. The secreted form of GPC3 was detected at a high concentration of about 1 μg/mL with a combination of the antibodies binding to the N-terminal side (Fig. 4). The concentration detected with a combination of the antibodies binding to the C-terminal side was low, suggesting that the N-terminal fragment was dominantly present in the secreted form of GPC3.

Subsequently, the culture supernatant of HepG2 was immunoprecipitated using an anti-GPC3 antibody to detect the secreted form of GPC3. In the case where M10D2 that binds to the N-terminal fragment was used, the secreted form of GPC3 of 40 kDa was detected (Fig. 5). On the other hand, in the case where M1E7 that binds to the C-terminal fragment was used, the secreted form of GPC3 was not detected. The immunoprecipitation test was carried out for all the obtained GPC3 antibodies. Every antibody binding to the N-terminal fragment strongly detected the secreted form of GPC3, while the secreted form of GPC3 was not detected or was weakly detected with the use of the antibodies binding to the C-terminal fragment (Fig. 16). The antibody that can detect the secreted form of GPC3 by immunoprecipitation is expected to be useful as an antibody for diagnosing hepatoma. In addition, the antibody that can hardly detect the secreted form

of GPC3 is expected to be useful in the development of a therapeutic antibody having an ADCC activity and a CDC activity, because such an antibody may migrate to the hepatoma lesion without being trapped in the secreted form of GPC3 present in the blood.

Example 13

Cloning of variable region of anti-GPC3 antibody

A variable region of the anti-GPC3 antibody was amplified by the RT-PCR method using the total RNA extracted from an anti-GPC3 antibody- producing hybridoma. The total RNA was extracted from 1 x 10^7 cells of the hybridoma with the use of RNeasy Plant Mini Kits (QIAGEN). By using 1 μg of the total RNA, the 5'-terminal gene fragment was amplified with the use of a SMART RACE cDNA Amplification Kit (CLONTECH) and any of the following synthetic oligonucleotides:

a synthetic oligonucleotide MHC-IgG1 complementary to the sequence of a mouse IgG1 constant region:

GGG CCA GTG GAT AGACAG ATG (SEQ ID NO: 7);

a synthetic oligonucleotide MHC-IgG2a complementary to the sequence of a mouse IgG2a constant region:

CAG GGG CCA GTG GAT AGA CCG ATG (SEQ ID NO: 8);

a synthetic oligonucleotide MHC-IgG2b complementary to the sequence of a mouse IgG2b constant region:

CAG GGG CCA GTG GAT AGA CTG ATG (SEQ ID NO: 9); and

a synthetic oligonucleotide kappa complementary to the sequence of a mouse kappa chain constant region: GCT CAC TGG ATG GTG GGA AGA TG (SEQ ID NO: 10).

A reverse transcription reaction was carried out at 42°C for 1 hour and 30 minutes. The PCR mixture (50 μL) contained 5 μL of 10x Advantage 2 PCR buffer, 5 μL of 10x Universal Primer A Mix, 0.2 mM dNTPs (dATP, dGTP, dCTP and dTTP), 1 μL of Advantage 2 Polymerase Mix (all from CLONTECH), 2.5 μL of the reverse transcription reaction product and 10 pmol of the synthetic oligonucleotide MHC-IgG1, MHC-IgG2a, MHC-IgG2b or kappa. PCR was carried out with 5 cycles consisting of 94°C for 30 seconds, 94°C for 5 seconds and 72°C for 3 minutes, 5 cycles consisting of 94°C for 5 seconds, 70°C for 10 seconds and 72°C for 3 minutes, and 25 cycles consisting of 94°C for 5 seconds, 68°C for 10 seconds and 72°C for 3 minutes. Lastly, the reaction product was heated at 72°C for 7 minutes. Each PCR product was purified from the agarose gel using a QIAquick Gel Extraction Kit (QIAGEN), cloned into pGEM-T Easy vector (Promega), and the nucleotide sequence was determined.

The nucleotide sequences of the H chain variable regions of M3C11, M13B3, M1E7, M3B8, M11F1, M19B11, M6B1, M18D4, M5B9, M10D2 and L9G11 are shown in SEQ ID NOs: 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 and 21, respectively, the amino acid sequences thereof are shown in SEQ ID NOs: 22, 23, 24, 25, 26, 27, 28, 29, 30, 31 and 32, respectively. The nucleotide

sequences of the L chain variable regions thereof are shown in SEQ ID NOs: 33, 34, 35, 36, 37, 38, 39, 40, 41, 42 and 43, respectively, and the amino acid sequences thereof are shown in SEQ ID NOs: 44, 45, 46, 47, 48, 49, 50, 51, 52, 53 and 54, respectively.

Example 14

Epitope classification using GST-fusion protein

To carry out a detail analysis of the epitopes for the antibodies binding to the C-terminal fragment, fusion proteins of successively shortened C-terminal peptides of GPC3 with GST, namely GC-1 (from Ser495 to Lys563), GC-2 (from Gly510 to Lys563), GC-3 (from Ala524 to Lys563), GC-4 (from Gly537 to Lys563) and GC-5 (from Ser550 to Lys563) were prepared. The C-terminal region of GPC3 was cloned into pGEX-4T-3 (Amersham) to construct a plasmid DNA in which the C-terminal region of GPC3 is ligated to the C-terminal side of GST. The plasmid DNA was introduced into DH5α, whereby a transformant was obtained. Then, IPTG was added at 1 mM to a culture of the transformant in the logarithmic growth phase to induce the expression of a GST-fusion protein . The bacterial cells were collected after 2 hours cultivation. The cells were homogenized by sonication, and centrifuged at 35,000 rpm for 30 minutes with XL-80 ultracentrifuge (Beckman, 70.1 Ti rotor). Then, the culture supernatant was recovered and purified with

GST Purification Modules (Amersham). The thus purified GST-fusion proteins were separated by SDS-PAGE under reducing conditions, and Western blotting was carried out with the anti-GPC3 antibodies (Fig. 6). M3C11 and M1E7 detected GC-1 and GC-2, while they did not detect GC-3, GC-4 and GC-5, indicating that the epitopes of these antibodies are contained in the region of GC-2, and that the region of GC-3 is not sufficient. M3B8 and M11F1 detected GC-1 GC-2, GC-3 and GC-4, while they did not detect GC-5, indicating that the epitopes of these antibodies are contained in the region of GC-4, and that the region of GC-5 is not sufficient. The minimum region of the GST-fusion protein to which each antibody can bind is listed in the column headed "Western blotting" of Fig. 16.

Example 15

Preparation of anti-GPC3 mouse-human chimeric antibody

The sequences of the H chain and the L chain variable regions of the anti-GPC3 antibodies were ligated to the sequences of a human IgG1 and a kappa chain constant regions. PCR was carried out by using a synthetic oligonucleotide, which is complementary to the 5'-terminal nucleotide sequence of the H chain variable region of each antibody and has a Kozak sequence, and a synthetic oligonucleotide, which is complementary to the 3'-terminal nucleotide sequence and has a NheI site. The obtained PCR product was cloned into pB-CH

vector in which a human IgG1 constant region was inserted into pBluescript KS(+) vector (Toyobo). The mouse H chain variable region and the human H chain (γ1 chain) constant region are ligated via the NheI site. The prepared H chain gene fragment was cloned into an expression vector, pCXND3. On the other hand, PCR was carried out by using a synthetic oligonucleotide, which is complementary to the 5'-terminal nucleotide sequence of the L chain variable region of each antibody and has a Kozak sequence, and a synthetic oligonucleotide, which is complementary to the 3'-terminal nucleotide sequence and has a BsiWI site. The obtained PCR product was cloned into pB-CL vector in which the human kappa chain constant region was inserted into pBluescript KS(+) vector (Toyobo). The human L chain variable region and the constant region are ligated via the BsiWI site. The prepared L chain gene fragment was cloned into an expression vector, pUCAG. This pUCAG vector was obtained by cloning a 2.6 kbp fragment obtained by digesting pCXN (Niwa et al., Gene 1991; 108: 193-200) with a restriction enzyme BamHI into the BamHI site of pUC19 vector (Toyobo).

To prepare an expression vector for an anti-GPC3 mouse-human chimeric antibody, a gene fragment was obtained by digesting the pUCAG vector containing the L chain gene fragment with a restriction enzyme HindIII (Takara Shuzo),and cloned into the HindIII site of the pCXND3 containing the H chain gene. This plasmid will express a neomycin-resistance

gene, DHFR gene and an anti-GPC3 mouse-human chimeric antibody in an animal cell.

A CHO cell line (DG44 cell line) stably expressing the antibody was prepared as follows. The gene was introduced into the cells by the electroporation method using Gene Pulser II (Bio-Rad). A mixture obtained by mixing 25 μg of the expression vector for each anti-GPC3 mouse-human chimeric antibody and 0.75 mL of a solution of CHO cells suspended in PBS (1×10^7 cell/mL) was cooled on ice for 10 minutes, and transferred to a cuvette. Then, a pulse was applied at 1.5 kV and a capacitance of 25 μFD. After a 10-minute recovery period at room temperature, the electroporated cells were suspended in 40 mL of CHO-S-SFM II medium (Invitrogen) containing 1x HT supplement (Invitrogen). The suspension was diluted to 50-fold with the same medium, and dispensed to a 96-well culture plate at 100 μL/well. After a 24-hour culture in a CO_2 incubator (5% CO_2), Geneticin (Invitrogen) was added at 0.5 mg/mL and the cells were cultured for 2 weeks. Tthe culture supernatant was taken from the well having a Geneticin resistant transformed cell colony and the amount of IgG was measured by the concentration determination method described below. A high-producing cell line was successively expanded to obtain a cell line that stably expresses an anti-GPC3 mouse-human chimeric antibody. The cell line was cultured at a large-scale and the culture supernatant was collected. The purification of each anti-GPC3

mouse-human chimeric antibody was carried out using Hi Trap ProteinG HP (Amersham).

Example 16

Measurement of complement-dependent cytotoxicity activity (CDC activity)

16.1 Preparation of human albumin veronal buffer (HAVB)

In milli-Q water, 12.75 g of NaCl (highest grade, Wako Pure Chemicals), 0.5625 g of Na-Barbital (highest grade, Wako Pure Chemicals) and 0.8625 g of Barbital (highest grade, Wako Pure Chemicals) were dissolved to a final volume of 200 mL and autoclaved at 121°C for 20 minutes. Then, 100 mL of autoclaved hot milli-Q water was added. The pH was 7.43 (recommended pH: 7.5). The solution was used as a 5x veronal buffer. In 50 mL of milli-Q water, 0.2205 g of $CaCl_2 \cdot 2H_2O$ (highest grade, Junsei Chemical) was dissolved to a final concentration of 0.03 mol/L, which was used as a $CaCl_2$ solution. In 50 mL of milli-Q water, 1.0165 g of $MgCl_2 \cdot 6H_2O$ (highest grade, Junsei Chemical) was dissolved to a final concentration of 0.1 mol/L, which was used as a $MgCl_2$ solution. In milli-Q water, 100 mL of the 5x veronal buffer, 4 mL of human serum albumin (25% Buminate (registered trademark), the concentration of human serum albumin: 250 mg/mL, Baxter Healthcare), 2.5 mL of the $CaCl_2$ solution, 2.5 mL of the $MgCl_2$ solution, 0.1 g of KCl (highest grade, Junsei Chemical) 0.5 g of glucose (D(+)-glucose, anhydrous glucose,

highest grade, Wako Pure Chemicals) were dissolved to a final volume of 500 mL, which was used as HAVB. After filter sterilization, the HAVB was stored at a preset temperature of 5°C.

16.2 Preparation of target cell

The CHO cell expressing full-length human GPC3 prepared in Example 4 was cultured in α-MEM medium containing nucleic acid (+) (GIBCO) supplemented with 10% FBS and 0.5 mg/mL Geneticin (GIBCO). The cells were dissociated from the dish using a cell dissociation buffer (Invitrogen Corp), dispensed to each well of a 96-well flat-bottomed plate (Falcon) at 1 x 10^4 cells/well, and cultured for 3 days. After the cultivation, 5.55 MBq of chromium-51 was added, and the cells were cultured in a 5% carbon dioxide gas incubator at 37°C for 1 hour. These cells were washed with HAVB twice, and 50 μL of HAVB was added and used as a target cell.

16.3 Chromium release test (CDC activity)

Each chimeric antibody was diluted with HAVB to make a 40 μg/mL antibody solution. To the target cell, 50 μL of each antibody solution was added, and left on ice for 15 minutes. Subsequently, to each well, 100 μL of the human serum from the peripheral blood of a healthy volunteer, which had been diluted with HAVB, was added to a final concentration of 25% (the final

concentration of antibody: 10 μg/mL), and left in a 5% carbon dioxide gas incubator at 37°C for 90 minutes. After the plate was centrifuged, 100 μL of the supernatant was collected from each well, the radioactivity was measured using a gamma counter. The specific chromium release rate was obtained by the following formula.

Specific chromium release rate (%) = (A-C) x 100/(B-C)

"A" represents the radioactivity (cpm) in each well, "B" represents the mean value of the radioactivities (cpm) in the wells in which 100 μL of 2% NP-40 aqueous solution (Nonidet P-40, Code No. 252-23, Nacalai Tesque) and 50 μL of HAVB were added to the target cell, and "C" represents the mean value of the radioactivities (cpm) in the wells in which 150 μL of HAVB was added to the target cell. The test was carried out in triplicate and the mean value and the standard deviation were calculated for CDC activity (%).

The results are shown in Fig. 7. Among 9 types of the anti-GPC3 chimeric antibodies, M3B8 and M11F1, which are an antibody recognizing the C-terminal side, showed a strong CDC activity against the CHO cell expressing GPC3, however, the CDC activity was not observed in the other antibodies. M3B8 and M11F1 belong to the group called "b" based on the competitive ELISA, and an important epitope for showing a strong CDC activity could be found.

Example 17

Measurement of ADCC activity using PBMC derived from human peripheral blood

17.1 Preparation of human PBMC solution

The heparinized peripheral blood obtained from a healthy volunteer was diluted to 2-fold with PBS(-), and overlayered on Ficoll-Paque TM PLUS (Amersham). After centrifugation at 500 x g for 30 minutes at 20°C, the middle layer, which is the mononuclear leukocyte fraction, was collected. The cells were washed 3 times, suspended in 10% FBS/RPMI and used as a human PBMC solution.

17.2 Preparation of target cell

The HepG2 cells cultured in 10% FBS/RPMI 1640 medium were dissociated from the dish using Trypsin-EDTA (Invitrogen), dispensed to each well of a 96-well U-bottomed plate (Falcon) at 1 x 10^4 cells/well, and cultured for 2 days. The CHO cell expressing full-length human GPC3 prepared in Example 4 was cultured in α-MEM nucleic acids (+) medium (GIBCO) supplemented with 10% FBS and 0.5 mg/mL Geneticin (GIBCO). The cells were dissociated from the dish using a cell dissociation buffer (Invitrogen Corp), dispensed to each well of a 96-well flat-bottomed plate (Falcon) at 1 x 10^4 cells/well, and cultured for 3 days. Chromium-51 (5.55 MBq) was added to each cell and the cells were cultured in a 5% carbon dioxide gas incubator

at 37°C for 1 hour. These cells were washed with the medium once, and 50 μL of 10% FBS/RPMI 1640 medium was added and used as a target cell.

17.3 Chromium release test (ADCC activity)

To the target cell, 50 μL of an antibody solution prepared at different concentrations was added, and reacted on ice for 15 minutes. Subsequently, 100 μL of the human PBMC solution was added at 5 x 10^5 cells/well, and cells were cultured in a 5% carbon dioxide gas incubator at 37°C for 4 hours. After the cultivation, the plate was centrifuged, and the radioactivity in 100 μL of the culture supernatant was measured using a gamma counter. The specific chromium release rate was obtained by the following formula.

specific chromium release rate (%) = (A-C) x 100/(B-C)

"A" represents the mean value of the radioactivities (cpm) in each well, "B" represents the mean value of the radioactivities (cpm) in the wells in which 100 μL of 2% NP-40 aqueous solution (Nonidet P-40, Code No. 252-23, Nacalai Tesque) and 50 μL of 10% FBS/RPMI medium were added to the target cell, and "C" represents the mean value of the radioactivities (cpm) in the wells in which 150 μL of 10% FBS/RPMI medium was added to the target cell. The test was carried out in triplicate and the mean value and the standard deviation were calculated for ADCC activity (%). The results are shown in Fig. 8. Among 9 types

of the anti-GPC3 chimeric antibodies, the antibodies recognizing the C-terminal side had a tendency of showing a strong ADCC activity.

Example 18

Immunization with GC-3 and selection of hybridoma

Among the obtained anti-GPC3 antibodies, only M11F1 and M3B8 showed a strong CDC activity, indicating that the CDC activity is epitope dependent. To obtain an antibody having both ADCC activity and CDC activity, a GST-fusion protein containing the epitope for M11F1 and M3B8, referred to as GC-3, was used for immunization. A large mount of GC-3 was purified by the above-mentioned method. The buffer was changed to PBS by gel filtration using Superdex 75 (Amersham). The obtained product was used as immunoprotein. using three Balb/c mice (purchased from Charles River Japan, Inc.) and three MRL/lpr mice were immunized with GC-3 in accordance with the above-mentioned method. For the first immunization, GC-3 was prepared at 100 μg/head and emulsified using FCA, which was subcutaneously administered. Two weeks later, GC-3 was prepared at 50 μg/head and emulsified using FIA, which was subcutaneously administered. After the fifth immunization, the final immunization (50 μg/head) was carried out for all mice by intravenously administering the immunoprotein via the tail. After cell fusion, hybridoma were screened by an ELISA

using an immunoplate coated with the soluble form of GPC3 core protein. A positive clone was monocloned by the limiting dilution method. As a result, 5 clones of antibodies (GC199, GC202, GC33, GC179 and GC194) that have a strong binding activity against GPC3 were obtained.

The antibody was purified from the culture supernatant of the hybridoma using Hi Trap proteinG HP, and analyzed in accordance with the above-mentioned method. The EC50 value was calculated by an ELISA using an immunoplate coated with the soluble form of GPC3 core protein, and the X-mode value of histogram at 5 μg/mL was measured by flow cytometry (Fig. 17). According to the epitope classification by a competitive ELISA, the antibodies were classified into the group b (GC199, GC202 and GC33) and a new epitope group f (GC179 and GC194). The epitope classification using the GST-fusion proteins indicated that GC199, GC202 and GC33 detected GC-1, GC-2, GC-3 and GC-4, but did not detect GC-5, suggesting that the epitopes for these antibodies are contained in the region of GC-4 in the same manner as the epitopes for M11F1 and M3B8, and that the region of GC-5 is not sufficient. On the other hand, GC179 and GC194 detected GC-1, GC-2 and GC-3, but did not detect GC-4 and GC-5, suggesting that the epitopes for these antibodies are contained in the region of GC-3, and that the region of GC-4 is not sufficient. The minimum region of the GST-fusion protein to which each antibody can bind is listed in the column

headed "Western blotting" of Fig. 17.

The H chain and the L chain variable regions of GC199, GC202, GC33, GC179 and GC194 were cloned in accordance with the above-mentioned method, and their sequences were determined. As for the L chain of GC194, 2 types of sequences were cloned. The nucleotide sequences of the H chain variable regions of GC199, GC202, GC33, GC179 and GC194 are shown in SEQ ID NOs: 55, 56, 57, 58 and 59, respectively, and the amino acid sequences thereof are shown in SEQ ID NOs: 60, 61, 62, 63 and 64, respectively. The nucleotide sequences of the L chain variable regions of GC199, GC202, GC33, GC179, GC194(1) and GC194(2) are shown in SEQ ID NOs: 65, 66, 67, 68, 69 and 70 respectively, and the amino acid sequences thereof are shown in SEQ ID NOs: 71, 72, 73, 74, 75 and 76, respectively.

Further, these amino acid sequences were examined for homology by comparing with the database of the amino acid sequences of known antibodies, whereby their CDR regions were determined as follows.

Antibody	CDR	Amino Acid Sequence	SEQ ID NO
M13B3(H)	CDR1	NYAMS	103
	CDR2	AINNNGDDTYYLDTVKD	104
	CDR3	QGGAY	105
M3B8(H)	CDR1	TYGMGVG	106
	CDR2	NIWWYDAKYYNSDLKS	107
	CDR3	MGLAWFAY	108
M11F1(H)	CDR1	IYGMGVG	109
	CDR2	NIWWNDDKYYNSALKS	110
	CDR3	IGYFYFDY	111

M5B9(H)	CDR1	GYWMH	112
	CDR2	AIYPGNSDTNYNQKFKG	113
	CDR3	SGDLTGGLAY	114
M6B1(H)	CDR1	SYAMS	115
	CDR2	AINSNGGTTYYPDTMKD	116
	CDR3	HNGGYENYGWFAY	117
M10D2(H)	CDR1	SYWMH	118
	CDR2	EIDPSDSYTYYNQKFRG	119
	CDR3	SNLGDGHYRFPAFPY	120
L9G11(H)	CDR1	SYWMH	118
	CDR2	TIDPSDSETHYNLQFKD	121
	CDR3	GAFYSSYSYWAWFAY	122
GC33(H)	CDR1	DYEMH	123
	CDR2	ALDPKTGDTAYSQKFKG	124
	CDR3	FYSYTY	125
GC179(H)	CDR1	INAMN	126
	CDR2	RIRSESNNYATYYGDSVKD	127
	CDR3	EVTTSFAY	128
GC194(H)	CDR1	ASAMN	129
	CDR2	RIRSKSNNYAIYYADSVKD	130
	CDR3	DPGYYGNPWFAY	131
GC199(H)	CDR1	DYSMH	132
	CDR2	WINTETGEPTYADDFKG	133
	CDR3	LY	134
GC202(H)	CDR1	TYGMGVG	106
	CDR2	NIWWHDDKYYNSALKS	135
	CDR3	IAPRYNKYEGFFAF	136

M13B3(L)	CDR1	KSSQSLLDSDGKTYLN	137
	CDR2	LVSKLDS	138
	CDR3	WQGTHFPLT	139
M3B8(L)	CDR1	KASQDINNYLS	140
	CDR2	RANRLVD	141
	CDR3	LQCDEFPPWT	142
M11F1(L)	CDR1	RSSQSLVHSNGNTYLH	143
	CDR2	KVSNRFS	144
	CDR3	SQSTHVPWT	145
M5B9(L)	CDR1	RSSKSLLHSNGITYLY	146
	CDR2	QMSNLAS	147
	CDR3	AQNLELPYT	148
M6B1(L)	CDR1	KASQDINKNII	149
	CDR2	YTSTLQP	150
	CDR3	LQYDNLPRT	151
M10D2(L)	CDR1	RASHSISNFLH	152
	CDR2	YASQSIS	153
	CDR3	QQSNIWSLT	154
L9G11(L)	CDR1	RASESVEYYGTSLMQ	155
	CDR2	GASNVES	156
	CDR3	QQSRKVPYT	157
GC33(L)	CDR1	RSSQSLVHSNGNTYLH	143
	CDR2	KVSNRFS	144
	CDR3	SQNTHVPPT	158
GC179(L)	CDR1	KSSKSLLHSNGNTYLN	159
	CDR2	WMSNLAS	160
	CDR3	MQHIEYPFT	161
GC194(L)1	CDR1	RSSKSLLHSYDITYLY	162
	CDR2	QMSNLAS	147
	CDR3	AQNLELPPT	163
GC194(L)2	CDR1	SASSSVSYMY	164
	CDR2	DTSNLAS	165
	CDR3	QQWSSYPLT	166
GC199(L)	CDR1	KSSQSLLHSDGKTFLN	167
	CDR2	LVSRLDS	168
	CDR3	CQGTHFPRT	169
GC202(L)	CDR1	RSSQSIVHSNGNTYLE	170
	CDR2	KVSNRFS	144
	CDR3	FQGSHVPWT	171

Example 19

Measurement of ADCC activity using mouse bone marrow derived effector cell

19.1 Preparation of mouse bone marrow derived effector cell solution

Bone marrow cells were collected from the femur of an SCID mouse (CLEA Japan, Inc., male, 10 weeks old), and suspended in 10% FBS/RPMI 1640 medium at 5 x 10^5 cells/mL. Mouse GM-CSF (PeproTech) and human IL-2 (PeproTech) were added at 10 ng/mL and 50 ng/mL, respectively, and the cells were cultured in a 5% carbon dioxide gas incubator at 37°C for 5 days. After the cultivation, the cells were scraped off with a scraper and washed with the medium once. Then, the cells were suspended in 10% FBS/RPMI 1640 medium at 5 x 10^6 cells/mL, and used as a mouse bone marrow derived effector cell solution.

19.2 Preparation of target cell

A human hepatoma cell line, HuH-7, was maintained and subcultured with DMEM medium (SIGMA) containing 10% FBS (ThermoTrace). The cells were dissociated from the dish using Cell Dissociation Buffer (Invitrogen), dispensed to each well of a 96-well U-bottomed plate (Falcon) at 1 x 10^4 cells/well, and cultured for 1 day. After the cultivation, 5.55 MBq of chromium-51 was added, and the cells were cultured in a 5% carbon dioxide gas incubator at 37°C for 1 hour. These cells

were washed with the medium once, and 50 μL of 10% FBS/RPMI 1640 medium was added and used as a target cell.

19.3 Chromium release test (ADCC activity)

To the target cell, 50 μL of an antibody solution prepared at different concentrations was added, and reacted on ice for 15 minutes. Subsequently, 100 μL of the mouse bone marrow derived effector cell solution (5×10^5 cells/well) was added, and cells were cultured in a 5% carbon dioxide gas incubator at 37°C for 4 hours. After the cultivation, the plate was centrifuged, and the radioactivity in 100 μL of the culture supernatant was measured using a gamma counter. The specific chromium release rate was obtained by the following formula.

Specific chromium release rate (%) = (A-C) x 100/(B-C)

"A" represents the mean value of the radioactivities (cpm) in each well, "B" represents the mean value of the radioactivities (cpm) in the wells in which 100 μL of 2% NP-40 aqueous solution (Nonidet P-40, Code No. 252-23, Nacalai Tesque) and 50 μL of 10% FBS/RPMI medium were added to the target cell, and "C" represents the mean value of the radioactivities (cpm) in the wells in which 150 μL of 10% FBS/RPMI medium was added to the target cell. The test was carried out in triplicate and the mean value and the standard deviation were calculated for ADCC activity (%).

The results are shown in Fig. 9. It was revealed that

GC33 antibody shows an ADCC activity when the concentration of antibody is 0.1 μg/mL or higher, and shows stronger activity than GC199 antibody.

Example 20

Antitumor activity of GC33 antibody to mouse model transplanted with human hepatoma

20.1 Preparation of mouse model transplanted with human hepatoma

A human hepatoma cell line, HuH-7, was prepared at 5 x 10^7 cells/mL in a solution containing DMEM medium and MATRIGEL (BD Bioscience) at a ratio of 1:1. On the previous day, 100 μL of an anti-asialo GM1 antibody solution (Wako Pure Chemicals, one vial was dissolved with 1mL of distilled water for injection then added 4 mL of physiologic saline) was intraperitoneally administered to a SCID mouse (male, 5 weeks old, CLEA Japan, Inc.). The mouse was transplanted with 100 μL of the above-mentioned cell suspension (5 x 10^6 cells/mouse) subcutaneously in the abdominal area.

20.2 Preparation and administration of antibody

Starting from the day 20 after the cell transplantation, an antibody solution prepared on the day of administration at 0.5 mg/mL (group of administration of 5 mg/kg) and at 0.1 mg/mL (group of administration of 1 mg/kg) with PBS(-) was

administered to the mouse model transplanted with a human hepatoma cells at 10 mL/kg via the tail vein once a week for 3 weeks. As a negative control, PBS(-) (vehicle) was administered at 10 mL/kg via the tail vein once a week for 3 weeks in a similar manner. Both groups consisted of 6 mice each.

20.3 Evaluation of antitumor effect

The antitumor effect of GC33 antibody on the mouse model transplanted with human hepatoma cells was evaluated with the change in tumor volume with time and tumor weight at 1 week after the final administration. The tumor volume was calculated by the following formula.

Tumor volume = (major axis) x (minor axis) x (minor axis)/2

As shown in Fig. 10, a significant inhibition of tumor growth was observed in the GC33 antibody group compared with the vehicle group.

Consequently, GC33 was shown to have an antitumor effect on the mouse model transplanted with a human hepatoma cells.

Example 21

Preparation of GC33 mouse-human chimeric antibody

The H chain and the L chain of GC33 were amplified by PCR using a synthetic oligonucleotide, which is complementary to the 5'-terminal nucleotide sequences and has a Kozak

sequence and a HindIII site, and a synthetic oligonucleotide, which is complementary to the 3'-terminal nucleotide sequences and has a BamHI site. After digestion with HindIII and BamHI, the obtained PCR product was cloned into an expression vector, HEFgγ1, in which a human IgG1 constant region was inserted, and an expression vector, HEFgκ, in which a human kappa chain constant region was inserted (Sato et al., Mol Immunol. 1994; 371-381). The vectors were introduced into a CHO cell (DG44 cell line) in accordance with the above-mentioned method, and a stably expressing cell line was established. The antibody was purified from the culture supernatant using Hi Trap ProteinG HP (Amersham). The concentration of IgG in the culture supernatant was measured by a human IgG sandwich ELISA using goat anti-human IgG (BIOSOURCE) and goat anti-human IgG alkaline phosphatase conjugate (BIOSOURCE), and the concentration was determined by the comparison with a commercially available human IgG (Cappel).

Example 22

Measurement of CDC activity and ADCC activity using GC33 mouse-human chimeric antibody

In accordance with the methods described in Examples 16 and 17, the CDC activities and ADCC activities of GC33, M3C11 and M1E7 mouse-human chimeric antibodies were measured. As for the target cell, the CHO cell expressing full-length GPC3

was used for measuring the CDC activity and HepG2 was used for measuring the ADCC activity. The results are shown in Fig. 11 and Fig. 12, respectively. It was revealed that, in either test system, GC33 shows a strong CDC activity and ADCC activity compared with the other two antibodies.

Example 23

Epitope analysis for GC33

To determine the epitope for GC33 in detail, fusion proteins of a further shorter C-terminal peptide of GPC3 and GST were prepared, and analyzed by Western blotting. The prepared GPC3-derived peptide sequences contained in the GST-fusion protein are shown in Fig. 13. Since GC33 can bind to GC-4 (aa 537-563), but cannot bind to GC-5 (aa 550-563), it was considered that the epitope is located in a region containing at least part of the aa 537-550 region. First, the peptides GC-6 (G N S Q Q A T P K D N E I S (SEQ ID NO: 93)), GC-7 (G N S Q Q A T P (SEQ ID NO: 94)), GC-8 (Q Q A T P K D N (SEQ ID NO: 95)) and GC-9 (T P K D N E I S (SEQ ID NO: 96)) were prepared. A forward oligo DNA and a reverse oligo DNA were prepared which were designed in such a manner that the cleavage site of EcoRI recognition sequence is attached to the 5' end and the cleavage site of SalI recognition sequence is attached to the 3' end, respectively. The synthesis of the oligo DNAs was done by Espec Oligo Service. The DNA was

purified with C-18 cartridge, phosphorylated at the 5' end and used for the analysis. Twenty-five microliters of the forward oligo DNA (10 μM) and 25 μL of the reverse oligo DNA (10 μM) were mixed and reacted at 94°C for 5 minutes, at 37°C for 10 minutes, and at room temperature for 15 minutes, then left at 4°C for 10 minutes to anneal the forward oligo DNA and the reverse oligo DNA. The concentration of the oligos was determined by the absorbance measurement at the molar ratio of the insert to the vector of 3:1. The oligos were cloned into EcoRI- and SalI-digested pGEX4T-3, and the nucleotide sequence was confirmed. A GST-fusion protein was prepared in accordance with the above-mentioned method, and purified using Gluthatione Sepharose 4B. The purified proteins were separated by SDS-PAGE under reducing conditions, and analyzed by Western blotting using GC33. As a result, the antibody GC33 could not detect any GST-fusion protein strongly, suggesting that a longer sequence at the C-terminal side is needed for the binding of GC33 (Fig. 14). Based on the above prediction, GC-11 (A T P K D N E I S T (SEQ ID NO: 97)), GC-12 (P K D N E I S T F H (SEQ ID NO: 98)), GC-13 (D N E I S T F H N L (SEQ ID NO: 99)) and GC-14 (E I S T F H N L G N (SEQ ID NO: 100)) were prepared and evaluated in the same manner. As a result, GC-11, GC-12 and GC-13 bound to GC33 more strongly, suggesting that the epitope for GC33 is located in the sequence from 544th to 553rd (P K D N E I S T F H) at the C-terminus of GPC3.

Example 24

Humanization of GC33

Antibody sequence data were obtained from publicly disclosed Kabat Database (ftp://ftp.ebi.ac.uk/pub/databases/kabat/) and from ImMunoGeneTics Database (IMGT). The H chain variable region and the L chain variable region were separately subjected to homology search. As a result, the H chain variable region was found to have a high homology with DN13 (Smithson et al., Mol Immunol. 1999; 36: 113-124), and the L chain variable region was found to have a high homology with homo sapiens IGK mRNA for immunoglobulin kappa light chain VLJ region, partial cds, clone: K64 of the accession number of AB064105. The signal sequence of the accession number of S40357 that has a high homology with AB064105 was used as a signal sequence of the L chain. The complementarity determining region (hereinafter referred to as CDR) of GC33 were transplanted into the frame work regions (hereinafter referred to as FR) of these human antibodies to prepare a humanized antibody.

Specifically, synthetic oligo DNAs of approximately 50 bases were designed in such a manner that approximately 20 bases of them were hybridized and these synthetic oligo DNAs were assembled together by the PCR method to prepare genes encoding each of the variable regions. They were digested at the HindIII

site inserted in the end of the 5'-terminal synthetic oligo DNA and the BamHI site inserted in the end of the 3'-terminal synthetic oligo DNA. The fragments were cloned into an expression vector, HEFgγ1, in which a human IgG constant region was cloned, or an expression vector, HEFgκ1, in which a human kappa chain constant region was cloned (Sato et. al., Mol Immunol. 1994; 371-381). The H chain and the L chain of the humanized GC33 constructed as above were named ver.a, respectively. The binding activity of the humanized GC33, whose H chain and the L chain were both ver.a (ver.a/ver.a), was lower than that of an antibody with mouse GC33 variable regions (mouse/mouse). Antibodies were constructed in which the mouse GC33 sequence and the ver.a sequence were chimerically combined (mouse/ver.a, ver.a/mouse) with regard to the H chain and the L chain, and their binding activities were evaluated. As a result, a decrease in binding activity was observed in ver.a/mouse, indicating that the decrease in binding activity due to amino acid replacement was attributed to the H chain (Fig. 15). Then, modified H chains, ver.c, ver.f, ver.h, ver.i, ver.j, ver.k were prepared. All these humanized GC33 showed a binding activity equivalent to that of a chimeric antibody having the mouse GC33 variable region (Fig. 15). The nucleotide sequences of the humanized GC33 H chain variable regions, ver.a, ver.c, ver.f, ver.h, ver.i, ver.j, ver.k were shown in SEQ ID NOs: 77, 78, 79, 80, 81, 82 and 83, respectively,

and the amino acid sequences thereof were shown in SEQ ID NOs: 84, 85, 86, 87, 88, 89 and 90, respectively. The nucleotide sequence and the amino acid sequence of a humanized GC33 L chain variable region, ver.a are shown in SEQ ID NOs: 91 and 92, respectively. In humanized GC33 H chain variable regions ver.i, ver.j and ver.k, the 6th glutamic acid residue is replaced with glutamine residue. The heat stability of these antibodies was significantly increased.

Example 25

Modification of humanized GC33 L chain

As for the deamidation of protein, the reaction rate constant of deamidation is known to be dependent on the primary sequence. It is also known that Asn-Gly is particularly susceptible to deamidation (Rocinson et. al., Proc. Natl. Acad. Sci. USA 2001; 98; 944-949). As for Asn33 in the CDR1 of a humanized GC33 L chain ver.a shown in SEQ ID NO: 91, the primary sequence is Asn-Gly, which is predicted to be very susceptible to deamidation.

To evaluate the effect of deamidation of Asn33 on the binding activity, a modified antibody was prepared in which Asn33 was replaced with Asp. A point mutation was introduced using a Quick Change Site-Directed Mutagenesis Kit (Stratagene) was used. More specifically, 50 μL of a reaction mixture containing 125 ng of a sense primer (CTT GTA CAC AGT

GAC GGA AAC ACC TAT: SEQ ID NO: 172), 125 ng of an antisense primer (ATA GGT GTT TCC GTC ACT GTG TAC AAG: SEQ ID NO: 173), 5 μL of 10x reaction buffer, 1 μL of dNTP mix, 10 ng of HEFgκ into which a humanized GC33 L chain ver.a had been cloned and 1 μL of Pfu Turbo DNA Polymerase was subjected to PCR of 12 cycles consisting of 95°C for 30 seconds, 55°C for 1 minute and 68°C for 9 minutes. Subsequently, a restriction enzyme, DpnI, was added and digestion was carried out at 37°C for 2 hours, and the digested product was introduced into XL1-Blue competent cell attached to the kit, whereby a transformant was obtained. The variable region was cleaved out from the clone in which each mutation was properly introduced, and cloned into HEFgκ again. It was introduced into a COS7 cell using Fugene 6 (Roche) together with HEFgγ1, in which a humanized GC33 H chain ver.k had been cloned. The antibody transiently expressed in the cell was recovered from the culture supernatant. The concentration of antibody was determined by a sandwich ELISA using the anti-human IgG antibody. The binding activity of the modified antibody was evaluated by an ELISA using an immunoplate coated with the soluble form of GPC3 core protein. As shown in Fig. 18, the binding activity was lost in the modified antibody (N33D) in which Asn33 had been replaced with Asp, suggesting that the effect of the deamidation of Asn33 on the binding activity was significant.

As a method of suppressing deamidation of Asn33,

replacement of Gly34 with another amino acid has been reported (International Patent Application WO 03057881A1). In accordance with the above-mentioned method, G34 was replaced with any of 17 amino acids other than Cys and Met using a Quick Change Site-Directed Mutagenesis Kit to prepare a series of modified antibodies, namely, G34A, G34D, G34E, G34F, G34H, G34N, G34P, G34Q, G34I, G34K, G34L, G34V, G34W, G34Y, G34R, G34S and G34T. These antibodies were transiently expressed in COS7 cells, and the binding activity was evaluated using the culture supernatant. It was found that the binding activity is maintained even if G34 is replaced with another amino acid, except for Pro (G34P) and Val (G34V).

The amino acid sequences of the light chain CDR1 of the above-mentioned modified antibodies are shown in SEQ ID NO: 174 (G34A), SEQ ID NO: 175 (G34D), SEQ ID NO: 176 (G34E), SEQ ID NO: 177 (G34F), SEQ ID NO: 178 (G34H), SEQ ID NO: 179 (G34N), SEQ ID NO: 180 (G34T), SEQ ID NO: 181 (G34Q), SEQ ID NO: 182 (G34I), SEQ ID NO: 183 (G34K), SEQ ID NO: 184 (G34L), SEQ ID NO: 185 (G34S), SEQ ID NO: 186 (G34W), SEQ ID NO: 187 (G34Y), SEQ ID NO: 188 (G34R), SEQ ID NO: 189 (G34V) and SEQ ID NO: 190 (G34P), respectively. The amino acid sequences of the light chain variable regions of the above-mentioned modified antibodies are shown in SEQ ID NO: 191 (G34A), SEQ ID NO: 192 (G34D), SEQ ID NO: 193 (G34E), SEQ ID NO: 194 (G34F), SEQ ID NO: 195 (G34H), SEQ ID NO: 196 (G34N), SEQ ID NO: 197 (G34T),

SEQ ID NO: 198 (G34Q), SEQ ID NO: 199 (G34I), SEQ ID NO: 200 (G34K), SEQ ID NO: 201 (G34L), SEQ ID NO: 202 (G34S), SEQ ID NO: 203 (G34W), SEQ ID NO: 204 (G34Y), SEQ ID NO: 205 (G34R), SEQ ID NO: 206 (G34V) and SEQ ID NO: 207 (G34P), respectively.

The antibody of the present invention can be used as a cell growth inhibitor, an anticancer agent or an agent for diagnosis of cancers.

Example 26

Preparation of human hepatoma cell line (SK-03) expressing full-length human GPC3

To obtain a cell line for evaluating a biological activity of the anti-GPC3 antibodies, a human hepatoma cell line expressing full-length GPC3 was established.

One microgram of a full-length human GPC3 gene expression vector treated with Pvu I was mixed with 2μL of FuGENE (Roche) to allow for complex formation. The complex was added to SK-HEP-1 cells (purchased from ATCC) for gene introduction. After incubation in CO2 incubator for 24 hours, GPC3 expressing cells were selected using Dulbecco's MEM (D-MEM, SIGMA) containing Geneticin at a final concentration of 1 mg/mL and 10% FBS. The resulting Geneticin-resistant colonies were collected and cell cloning was carried out by the limiting dilution method. The expression of human GPC3 of each cell clone was assayed by flow cytometory using the chimeric

antibody GC33 and FITC-labeled goat anti-human IgG antibody (ICN). In this way, a stably expressing cell line SK-03 was obtained.

Example 27

Comparison of CDC activity and ADCC activity of mouse-human chimeric antibodies

In order to directly compare the CDC activity and ADCC activity of the mouse-human chimeric antibodies GC33, M3C11, and M1E7 described in Example 22, the CDC activity and ADCC activity of three antibodies were measured in the same test system according to the method described in Examples 16 and 17. As for the target cell, the CHO cell expressing full-length GPC3 was used for measuring the CDC activity and SK-03 was used for measuring the ADCC activity. The results are shown in Fig. 19 and Fig. 20, respectively. It was revealed that, in either test system, GC33 shows a stronger CDC activity and ADCC activity compared with the other two antibodies.

Industrial Applicability

The antibody of the present invention can be used as a cell growth inhibitor, an anticancer agent and an agent for diagnosis of cancers.